A STUDY OF THE PROTEINS OF NASCENT AND MATURE MAMMALIAN RIBOSOMES

by

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A study of the synthesis of the ribosomal particle is prompted by two major considerations: its central role in the complex process of protein synthesis, and its position as the smallest cellular organelle requiring a specific assembly of macromolecules.

J.R. Warner, 1966.

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ABBREVIATIONS

The standard abbreviations found in Biochem. J. (1972) 126,1. are used throughout this thesis. The following additional abbreviations are also found in the text:

EDTA	disodium salt of ethylene diamine tetra acetic acid
DNase	Deoxyribonuclease (EC 3.1.4.5.)
RNase	Ribonuclease (EC 2.7.7.16.)
PPLO	Pleuropneumonia-like organisms
SDS	Sodium dodecyl sulphate
rRNA	ribosomal RNA
rpreRNA	precursor of ribosomal RNA

CONTENTS

SUMMARY

I.

HeLa cell ribosomal subunits were characterised after dissociation with EDTA or 0.85M KCl. EDTA dissociated subunits, separated on sucrose gradients, showed minimal cross-contamination. Conversely, although the 40s KCl-dissociated subparticle was obtained pure from sucrose gradients, its 60s counterpart was contaminated to 7% with small subunit (40s) material. CsCl equilibrium centrifugation and isotopic studies of the subparticles derived by EDTA or KCl treatment indicated that although the respective small subunits had identical RNA/protein ratios, KCl dissociated large subunits contained approximately 30% less protein than their EDTA-derived counterpart. It was concluded that part of the protein component of the large ribosomal subunit is loosely associated with that particle and is readily stripped by increasing the ionic strength.

II.

45s and 32s RNA was extracted from HeLa cell nucleolar 80s and 55s particles and was shown to contain polynucleotide sequences destined to appear on (28s + 18s) and 28s rRNA respectively. The 55s particle, isolated on sucrose gradients, was pure and undegraded. Conversely, 80s material was unstable, and on centrifugation, underwent degradation to particles of sedimentation coefficient 56s and 27s. This degradation could be inhibited completely by treatment of the particles with glutaraldehyde. Recentrifugation of glutaraldehyde fixed 80s particles demonstrated that they were approximately 25% contaminated with 55s material. Modification of the method of nucleolar particle preparation and of the cells' environment prior to harvesting failed to reduce this degree of contamination and did not improve the yield of 80s material.

III.

A two dimensional fingerprinting technique was used to characterise the proteins of large and small ribosomal subunits and their putative nucleolar precursors in HeLa cells. It was found that:

(a) L-[^{35}S]methionine- or cystine-labelled HeLa cell large and small subunit peptide fingerprints were markedly and reproducibly different from each other; and patterns derived from HeLa monolayer or suspension cell ribosomal subunits presented the same characteristic features.

(b) Ribosome dissociation with EDTA or 0.85M KCl did not influence materially the fingerprint pattern for each subunit, nor did variations in the method of subunit protein extraction and processing affect the fingerprints obtained.

(c) 55s nucleolar particles contained most peptides found on 50s ribosomes, but no 30s peptides. 80s particles also contained most 50s peptides and, in addition, a few 30s peptides present in much less than molar amounts. On no occasion did the 80s particle peptide fingerprint demonstrate all characteristic 30s spots, although many such peptides were identifiable at the top of the sucrose gradient employed to isolate the nucleolar particles. It was concluded that 30s peptide deficiency on 80s fingerprints either reflected the true in vivo situation or resulted from the extraction procedures employed in the isolation of the particles. All "pulse-labelled" peptides on 80s and 55s nucleolar particles could be "chased" onto (50s + 30s) and 50s ribosomal subunits respectively, confirming the precursor-product relationship between these particles.

(d) Inhibition of ribosome maturation by the use of actinomycin D or toyocamycin did not prevent the specific association of certain cytoplasmic proteins with pre-existing large ribosomal subunits. The small ribosomal subunit did not participate in a similar particle-

protein interaction.

(e) The kinetics of appearance in the cytoplasm of ribosome-associated newly synthesised subunit proteins was investigated. After a "pulse" of [^{35}S]methionine, the first labelled proteins to appear on large ribosomal subunits were the "exchangeable" proteins described above. Radioactivity was not found in all 50s subunit proteins until after approximately 60 minutes' labelling, whereas all small subunit proteins were labelled within about 30 minutes.

IV.

Buoyant density studies of nucleolar and cytoplasmic ribonucleoprotein particles showed that there is progressive protein loss from the nucleolar precursors during their maturation to cytoplasmic ribosomal subunits. Moreover, there did not appear to be sequential protein addition to the 80s and 55s particles during their maturation in the nucleolus. On the contrary, an exchange of proteins was apparent between these particles and a nucleolar protein "pool". Furthermore, a 70s particle was found to be a transient intermediate in the 80s to 55s ribonucleoprotein conversion step.

V.

The two dimensional fingerprinting technique was used in a comparative study of the proteins of ribosomal subunits derived from a number of different eukaryotic species.

VI.

The results of this study, together with data from the literature, have permitted the formulation of a tentative mechanism of ribosome assembly in HeLa cells.

INTRODUCTION.

SECTION I - INTRODUCTION.

I A General

This study was undertaken to define a simple and rapid procedure of sufficient resolution to permit the characterisation of microgram amounts of complex protein mixtures. The procedure was used to investigate the protein moiety of mammalian ribosomal subunits, and to analyse the mechanism of ribosome assembly in animal cells. To this end, it was also desirable to re-examine the procedures described in the literature for isolation and purification of cytoplasmic and nucleolar ribonucleoprotein particles, and to utilise polypeptide fractionation techniques to determine the relative protein contents of particles prepared in various ways and from different sources.

The three dimensional conformation which the proteins and RNA of eukaryotic and prokaryotic ribosomes adopt in the active particle is not yet known, but recent advances in the study, particularly of prokaryotic ribosomes, suggest that a coherent structural picture of the ribosome may soon be forthcoming. Present evidence indicates that all ribosomes consist of two dissimilar subunits, a larger (sedimentation coefficient = 50-60s in different cells), and smaller (30-45s in different cells), which associate reversibly during protein synthesis (Falvey and Staehelin, 1970a). Each subunit contains approximately equal amounts of RNA and protein, and over recent years, intensive investigation of prokaryotic ribosomes has revealed the considerable structural complexity of these particles. Table I(1) (adapted from Maden, 1971) compares some properties of eukaryotic and prokaryotic ribosomes.

Because of the abundance of experimental observations on ribosome structure and formation, both in eukaryotes and prokaryotes, and the numerous recent review articles dealing with these topics (see Darnell, 1968; Maden,1968, 1971; Spirin and Gavrilova, 1969; Attardi and Amaldi, 1970; Nomura, 1970;

Table I (1). Some Physicochemical Parameters of E.coli and Mammalian Ribosomes

	E. coli			Mammalian		
	monomer	subunits		monomer	subunits	
Sedimentation coefficient (approx.)	70s	50s	30s (1)	80s	60s	40-45s (2)
Particle Weight	2.6×10^6	1.8×10^6	0.7×10^6 (1)	4.3×10^6	2.7×10^6 (2)	
Sedimentation coefficient of RNA		23s (5s)	16s		28s (5s)	18s (2)
Molec. weight of RNA		1.07×10^6	0.55×10^6 (3,4)		$(1.64 \times 10^6$ (5) $(1.9 \times 10^6$	0.67×10^6 (5) 0.7×10^6 (6)
Molec. weight of protein		5.2×10^5 (11)	2.6×10^5 (10)		1.4×10^6 (12)	$6.0 - 7.8 \times 10^5$ (12)
Methylation pattern of RNA	majority of methyl groups on bases (7)			majority of methyl groups on 2-O' ribose (8)		
RNA content	60%	63%	62% (1)	50% (9)		
Ease of dissociation into subunits	Dissociate at 10^{-4}M Mg^{2+}			Mg^{2+}-free buffer, EDTA or 0.85-1M KCl required		

References

(1) Tissieres et al. (1959)
(2) Spirin and Gavrilova (1969)
(3) Kurland (1960)
(4) Stanley and Bock (1965)
(5) Petermann and Pavlovec (1966)
(6) McConkey and Hopkins (1969)
(7) Fellner and Sanger (1968); Fellner (1969)
(8) Wagner et al. (1967)
(9) Calculated from Petermann and Pavlovec (1966)
(10) Hardy et al. (1969)
(11) Mora et al. (1971)
(12) Bickle and Traut (1971)

Burdon, 1971), this introductory account will be restricted to those aspects of ribosome structure and formation which are of particular relevance to the present study, namely:-

(a) a brief consideration of E. coli ribosomal proteins (Section I B) and

(b) an outline of mammalian ribosomal structure and formation (Section I C).

The E. coli ribosome is, from a structural standpoint, the best understood of all ribosomes, and indeed of all cell organelles. Careful extrapolation of results derived from this organism may facilitate an understanding of the eukaryotic system and suggest fruitful avenues of research into the structure of the eukaryotic ribosome. Moreover, some techniques derived from the investigation of prokaryotic ribosomal structure may be directly applicable to studies of the eukaryotic system. Despite the fact that present knowledge of the structure of the eukaryotic ribosome in comparison to its prokaryotic counterpart, is very incomplete, the relative ease of isolation of ribosomal precursors from eukaryotic nuclei has facilitated investigation of ribosome assembly in these cells. Integration of current knowledge of prokaryotic ribosome structure and eukaryotic ribosome assembly provides a basis for the synthesis of an experimental approach to the study of the mechanism of rRNA and ribosomal protein interaction during the formation of HeLa cell ribosomes.

I B Escherichia coli ribosomal proteins.

The prokaryotic ribosome has been shown to contain a heterogeneous population of proteins with a combined molecular weight of 8×10^5 daltons (see Table I (1)). The isolation of each protein in pure form from E. coli ribosomes has been achieved by a number of laboratories,and it has been shown that 21 distinct proteins exist on the small subunit (Wittmann et al.,1971),and 27-34 on the large (Traut et al.,1969; Kaltschmidt and Wittmann,1970a; Mora et al.,1971). Preliminary investigations by Traut et al. (1967) had suggested that considerable complexity and heterogeneity existed among the proteins of

E.coli ribosomal subunits. Their technique involved fingerprinting of tryptic digests of [^{35}S]sulphate-labelled unprefractionated E.coli ribosomes on paper, and locating the radioactive spots by autoradiography. Using this procedure, they demonstrated that a fairly large number of methionine-or cystine-containing peptides existed on each ribosomal subunit, indicating that the subunit proteins are heterogeneous. Moreover, by comparison of the patterns obtained for large and small subunits, it was evident that each subunit contains its own distinct protein set. Employment of this technique provided, at that time, valuable information regarding the structural proteins of the E.coli ribosome, although its usefulness in the study of prokaryotic ribosomal proteins has now been eclipsed by the development of more definitive protein fractionation procedures. Such procedures usually involve preliminary separation of ribosomal subunits on sucrose gradients. Extraction of the proteins from the intact ribosomal subunits may be achieved by the use of 66% acetic acid (Waller and Harris, 1961), lithium chloride/urea (Leboy et al., 1964), 2-chloroethanol (Fogel and Sypherd, 1968), or ribonuclease treatment in 6M urea (Otaka et al., 1968), and the isolated proteins are then purified by a variety of techniques including salt precipitation, ion-exchange column chromatography, gel filtration, preparative polyacrylamide gel electrophoresis, or a combination of these. This approach has permitted complete separation of all 21 proteins of the small ribosomal subunit (Wittmann et al.,1971) of E.coli, and a common nomenclature for these proteins, based upon the two dimensional gel electrophoresis system of Kaltschmidt and Wittmann (1970b) has been described (Table I (2)). Agreement is not complete regarding the protein complement of the large E.coli subunit. Traut and his co-workers (1969) isolated 36 proteins from the 50s subunit and concluded that the total number of proteins on that subunit could be between 34 and 38. By two dimensional electrophoresis, Kaltschmidt and Wittmann (1970b) demonstrated the presence of 34 proteins on the large subunit. Kurland's group (Mora et al., 1971), however,

Table I (2) Correlation of 30s ribosomal proteins studied in four different laboratories

Berlin Code (Wittman)	Uppsala Code (Kurland)	e	i	a	m	Madison Code (Nomura)	e	i	a	m	Geneva Code (Tissieres)	e	i	a	m
S1	1	+	+	+	+	P1	+				13			+	+
S2	4a	+	+	+	+	P2	+				11	+	+	+	+
S3	9(+5)	+	+	+	+	P3	+				10b	+	+	+	+
S4	10	+	+	+	+	P4a	+				9	+	+		+
S5	3	+	+	+	+	P4	+				8a	+	+	+	+
S6	2	+	+	+	+	P3b+P3c	+	+			10a	+	+	+	+
S7	8	+	+	+	+	P5	+				7	+	+	+	+
S8	2a	+	+	+	+	P4b	+				8b	+	+		+
S9	12	+	+	+	+	P8	+	+			5	+	+	+	+
S10	4	+	+	+	+	P6	+				6	+	+	+	+
S11	11	+	+	+	+	P7	+	+	+		4c	+	+	+	+
S12	15	+	+	+	+	P10	+								
S13	15b	+	+			P10a	+								
S14	12b	+	+	+	+	P11	+								
S15	14	+	+	+	+	P10b	+				4b	+	+	+	+
S16	6	+	+	+	+	(P9	+	+			4a			+	+
S17	7	+	+	+	+	(P9					3a	+	+		+
S18	12a	+	+	+	+	P12	+				2b	+	+		+
S19	13	+	+	+	+	P13	+				2a	+	+	+	+
S20	16	+	+	+	+	P14	+				1	+	+	+	+
S21	15a	+	+	+	+	P15	+				0				+

Correlation with S1-S21 were done by: e: two-dimensional polyacrylamide gel electrophoresis; i: immunological techniques, especially by Ouchterlony's double diffusion test; a: amino acid compositions; m: molecular weights.

have purified 27 50s proteins that yield unique tryptic peptide "maps" and amino acid compositions. They have identified 7 additional components either as artefacts produced by the purification procedure or as chemically modified proteins which appear after cold storage. They therefore conclude that there are at least 27 proteins in the 50s subunit of E.coli and not many more. At present, a comparison is being made between their proteins and those obtained by Wittmann.

Inconsistency in the number of proteins isolated from complex mixtures is not uncommon and may derive from one or more of three sources:-

(i) Some proteins may be non-ribosomal, bound non-specifically to the ribosome. For example, ribonuclease I exists outside the E.coli membrane in vivo (Neu and Heppel, 1964), but becomes bound to the 30s subunit on cell lysis (Elson and Tal, 1959; Waller, 1964).

(ii) Some polypeptides may be artefacts produced by chemical or enzymic modification of proteins in the course of their isolation.

(iii) Some fractionated proteins may, in fact, not be pure, but consist of a mixture of two or more proteins.

The first problem is the most difficult to resolve, and is related to the definition of a ribosomal protein. This definition may take one of three forms.

First, ribosomal proteins may be defined as those proteins which remain on the functionally intact ribosome after extensive purification. Various methods of purification have been used, including ammonium sulphate precipation (Kurland, 1966), centrifugation through discontinuous sucrose gradients, or washing in 0.5 or 1.0M ammonium chloride. However, it is possible that these procedures may still not be able to remove some tightly bound contaminating proteins.

Secondly, ribosomal proteins may also be defined as those polypeptides required to obtain functional ribosomes in an in vitro ribosome reconstitution system (see Traub and Nomura, 1968). Using this definition,

19 of the 21 proteins isolated from the ribosomal subunit were shown to be functionally necessary.

Thirdly, genetic criteria may be used to determine which proteins are ribosomal. When a protein is shown to be altered as the result of a mutation affecting a presumed ribosomal function, that protein can be considered to be a genuine ribosomal protein. Thus, the 30s protein S12, controlled by the rpxL locus (Birge and Kurland, 1969; Ozaki et al., 1969), and S5, controlled by the rpxE locus (Bollen et al., 1969) can be regarded as ribosomal proteins (see Wittmann et al., (1971) for nomenclature).

It is of interest to note that no protein has been found to be common to both subunits. This conclusion was reached after chemical (Strnad and Sypherd, 1969; Traut et al., 1969; Kurland, 1970) and immunological studies (Traut et al., 1969; Stöffler and Wittmann, 1971). Moreover, physical and immunological techniques have failed to show the presence of common peptides in the individual ribosomal proteins (Traut et al., 1969; Stöffler and Wittmann, 1971), although it has been reported (Möller et al., 1970; Mora et al., 1971) that one protein is duplicated on the 50s E.coli ribosomal subunit, and similar tryptic peptides appear, from the data of Mora et al. 1971), to occur on different 50s proteins.

Stoichiometric data are now available for E.coli ribosomal proteins. Kurland's group (Voynow and Kurland, 1971) have pointed out that the aggregate mass (410,000 daltons) of the small subunit proteins is inconsistent with the conclusion that there is one copy of each of these proteins in every small subunit, which contains only 260,000 daltons of protein (Hardy et al., 1969; Craven et al., 1969). Voynow and Kurland (1971), moreover, have shown that there are at least two stoichiometric classes of protein in the 30s subunit. One group is present in amounts corresponding to one copy per ribosome ("unit" proteins), and a second group in amounts much less than one copy per ribosome ("fractional" proteins), supporting

Table I (3) Stoichiometric and functional classification of E.coli 30s ribosomal proteins.

30s protein	Functional Class	Stoichiometric class
S1	Not known	(F)
S2	Dispensable	(F)
S3	Required for function	U
S4	Required for assembly	U
S5	Dispensable	(U)
S6	Not known	(U)
S7	Required for assembly	U
S8	Required for assembly	U
S9	Required for assembly	U
S10	Required for function	(U)
S11	Required for function	F
S12	Required for function	(U)
S13	Not known	(F)
S14	Required for function	F
S15	Dispensable	-
S16	Required for assembly	U
S17	Required for assembly	U
S18	Dispensable	(U)
S19	Required for function	F
S20	Dispensable	F
S21	Dispensable	F

U signifies a "unit" protein and F a "fractional" protein.
(U) and (F) indicate tentative stoichiometric classifications.

the conclusion that the purified ribosomes are structurally (and probably also functionally) heterogeneous. There are several possible explanations for the non-stoichiometry of the 30s ribosomal proteins:-

(i) Some fractional proteins may be non-ribosomal contaminants (e.g. ribonuclease I as described previously). Protein S1 may fall into this category since it is present only to the extent of 0.1 (Kurland et al., 1969) or 0.4 copy (Traut et al., 1969) per ribosome, and appears to be inessential for reconstitution of functionally active ribosomes (Nomura et al., 1969).

(ii) During protein isolation and purification, some proteins present in 1:1 stoichiometric amounts in vivo may be partially lost. However, attempts to find fractional ribosomal proteins in "post-ribosomal supernatants", or adsorbed to cell membrane fractions have been unsuccessful (Voynow and Kurland, 1971) and Stöffler (G. Stöffler, unpublished) has shown by an immunochemical assay that such material amounts to approximately 1% of the mass of ribosomal protein in the bacteria, a much lower figure than would be expected if serious ribosomal protein loss were occurring during isolation and purification procedures.

(iii) The observed heterogeneity of ribosomal populations may reflect the true situation in vivo. Voynow and Kurland's (1971) statement of the above conclusion is considerably strengthened by data of Nomura et al., (1969) who, by studying the contribution of most of the 30s proteins to the activity and structural integrity of the 30s particle, have found that each protein falls into one of two classes, depending on whether it is required for particle formation, or merely to permit particle mediated in vitro protein synthesising activity. Table I (3) summarises the findings of Nomura et al. (1969) and correlates them with the stoichiometric classification of Voynow and Kurland (1971). The relationship between "unit" proteins and those required for ribosome assembly is striking, and since none of the proteins which are required for assembly of the 30s particle have been identified as fractional proteins, the absence of certain (fractional)

proteins from some ribosomes presents no problem for the assembly of these ribosomes.

Two extreme views of the functional significance of the structural heterogeneity of E.coli 30s ribosomal subunits may be envisaged:-

(i) The Static Model

Here, the fractional proteins differentiate ribosome classes functionally. For example, one class of ribosomal subunits may initiate protein synthesis at the end of a polycistronic message while another class may commence synthesis at a more distal cistron.

(ii) The Dynamic Model

Here, by fractional protein exchange with the 30s subunit, a functional cycle may occur as the ribosome proceeds through the different stages of protein synthesis. Such an exchange of free ribosomal proteins with those of the intact ribosome has been described (Kurland et al., 1969) and makes the dynamic model worthy of further study.

In contrast to the 30s proteins, most E.coli large subunit proteins appear to be present in amounts corresponding to one copy per 50s particle, suggesting that these particles are homogeneous in the cell. Traut et al. (1969) found that 31 50s proteins exist in "unit" amounts and only 2-4 occur in amounts much less than one copy per ribosomal subunit. Kurland and co-workers (Mora et al., 1971) have also reported that the 50s subunit can accommodate one copy of each of the 27 proteins which they have isolated from it. However, they, and Müller et al. (1970) have discovered one complication. One protein appears to occur in amounts corresponding to at least two copies per particle. If more proteins of this sort are discovered, the homogeneity of the 50s subunit could become questionable, but the presence of such repeat structures would have great relevance to the function of the large subparticles.

I C Structure and Assembly of Mammalian Ribosomes.

Before describing in detail the current concept of mammalian ribosome assembly, a general account of the present understanding of mammalian ribosome

structure will be presented.

(1) Structure of Mammalian Ribosomes.

Animal cell ribosomes, in general, have a monomer sedimentation coefficient of 80s and subunit "S" values of 40s and 60s when isolated in the presence of magnesium ions (Martin and Wool, 1968; Hamilton, Pavlovec and Petermann, 1971). Dissociation by chelating agents (e.g. EDTA or pyrophosphate) produces subunits with sedimentation coefficients of approximately 50s and 30s (Hamilton and Petermann, 1959; Kuff and Ziegel, 1960; Lamfrom and Glowacki, 1962; Tashiro and Siekevitz, 1965; Girard et al., 1965), apparently as a result of partial subparticle unfolding (Gesteland, 1966; Tashiro and Morimoto, 1966; Spirin and Gavrilova, 1969). The structure of the ribosome is best described by considering, in turn, each of the chemical constituents (RNA and protein) of the particle:-

(a) Ribosomal RNA.

The mammalian ribosome contains three constituent RNA species - one with a sedimentation coefficient of approximately 18s, associated with the small subunit; and two (28s and 5s) found on the large subparticle (see Table I (1) and Maden, 1971; Burdon, 1971). Some eukaryotic large subunits also contain a small 7s RNA molecule which separates from the 28s rRNA by treatments which disrupt hydrogen bonds (Pene et al., 1968; Elicieri and Green, 1969). Pene et al. (1968) have presented evidence to indicate that 7s RNA is derived from the same polynucleotide precursor as its accompanying 28s molecule.

Biosynthesis of rRNA involves a transcriptional step and post-transcriptional modifications to the nascent RNA in all species. Recent work by Siddebottom and Harris (1969), using u.v. microirradiation to disrupt RNA synthesis and stability, confirmed earlier observations from cytochemical (Caspersson and Schultz, 1940; Brachet, 1942), autoradiographic (McMaster-Kaye and Taylor, 1958; Perry et al., 1961; Perry, 1962), and genetic studies (Brown and Gurdon, 1964) that the synthesis of eukaryotic rpreRNA

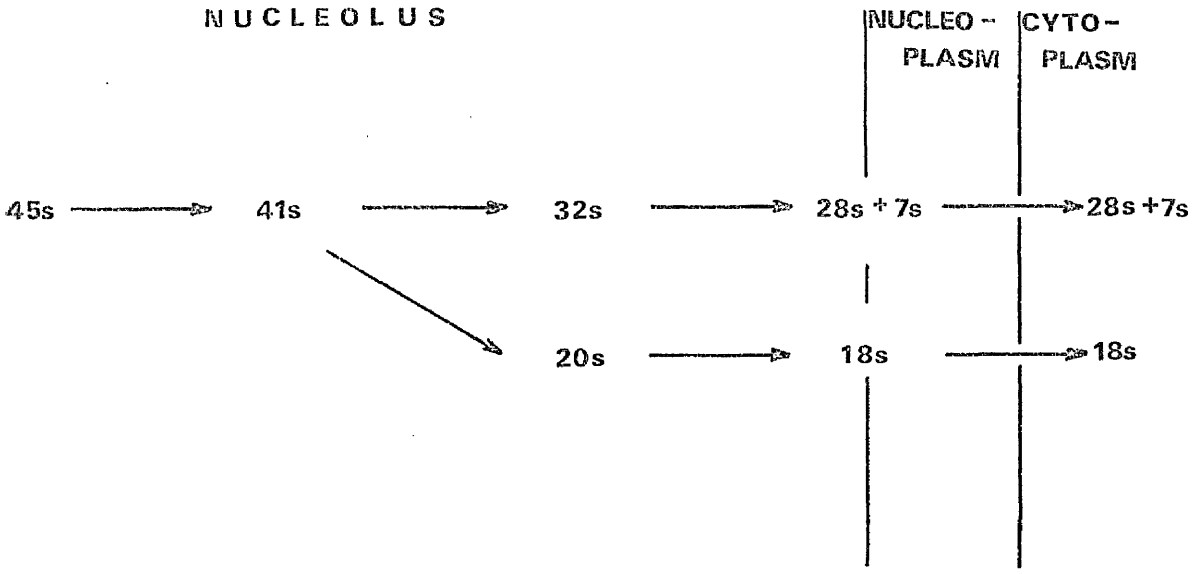


Fig. I (1)

Proposed scheme for the maturation of 45s nucleolar RNA to 28s and 18s rRNA's
(modified from Weinberg and Penman, 1970)

occurs in the nucleolus. The generally accepted mechanism of rpreRNA maturation to 28s and 18s RNA is indicated in Fig. I (1).

The development of techniques for separating eukaryotic cells into uncontaminated cytoplasmic, nucleolar, and nuclear supernatant fractions has facilitated determination of the origin and mechanism of maturation of both nascent rRNA and ribonucleoprotein particles. After incubation of HeLa cells with radioactive uridine (Penman, Smith and Holtzman, 1966) or methyl-methionine (Greenberg and Penman, 1966; Zimmerman and Holler, 1967), there appears in the nucleolus a labelled RNA molecule with sedimentation coefficient of 45s. With increasing "pulse" duration, the 45s RNA becomes more prominent, and after approximately 20 minutes, a labelled 32s RNA species can also be extracted from the nucleolus (Penman, Smith and Holtzman, 1966). Simultaneously, 18s rRNA is found, first in the nucleoplasm, and then in the cytoplasm; and ultimately, after 50-60 minutes labelling, radioactive 28s rRNA can be isolated from large ribosomal subunits in the cytoplasm (Penman, 1966; Penman, Smith and Holtzman, 1966; Vaughan, Warner and Darnell, 1967). A similar pattern of rpreRNA maturation has also been noted in amphibian oocytes (Gall, 1966; Rogers, 1968), insects (Edström and Daneholt, 1967) yeast (Retel and Planta, 1967) and germinating wheat (Chen et al., 1971).

In support of the tentative precursor-product relationships between 45s, 32, 18s and 28s RNA suggested by the above experimental data, it has been shown that 45s RNA has a base composition similar to that of rRNA (Scherrer, Latham and Darnell, 1963; Soeiro, Birnboim and Darnell, 1966). Furthermore, the methylation patterns of 32s and 28s RNA are identical and differ from that of 18s RNA, while the 45s pattern resembles that of an equimolar mixture of 28s and 18s rRNA (Wagner et al., 1967; Salim et al., 1970; Maden et al., 1972), although at least one methylation step seems to occur on 18s RNA after its maturation from the 45s molecule (Zimmerman, 1968; Maden et al., 1972). This methylation event would appear to occur on the 18s molecule itself since its 20s precursor (Fig. I (1)) is still evidently deficient in one methylated sequence (Maden et al., 1972).

Table I (4) Physical properties of some eukaryotic ribosomal proteins

Eukaryote	Large subunit protein weight	Small subunit protein content	No. of proteins large subunit	No. of proteins small subunit	$\overline{M}_w$ ($\times 10^{-3}$) L	$\overline{M}_w$ ($\times 10^{-3}$) S	$\overline{M}_n$ L	$\overline{M}_n$ S
Yeast (Warner, 1971)	1.05×10^6	0.57×10^6	50	29			21	19.6
Euglena (Bickle and Traut,1971)	1.2×10^6	0.75×10^6	39-46	27-32	31	28	26	32
Mouse plasmacytoma (Bickle and Traut,1971)	1.4×10^6	$0.6 - 0.78 \times 10^6$	42-52	18-27	32	34	27	29
Xenopus ovary (Ford,1971)	1.44×10^6	0.90×10^6	26-46	24-36	-	-	47.5	30 (mean)
Rabbit reticulocyte ribosome (King,Gould and Shearman, 1971)	1.09×10^6	0.64×10^6	37-61	30-40	25	21	22	18

$\overline{M}_w$ and $\overline{M}_n$ signify Weight Average and Number Average molecular weights respectively.

The methylation pattern of the 41s RNA species (Fig. I (1)) is identical to that of its 45s precursor, and contains the combined methylated sequences of 20s and 28s RNA (Maden et al., 1972). Finally, in confirmation of the assigned sites of origin of rRNA, Jeanteur and Attardi (1969) have shown by DNA-RNA hybridisation experiments that 28s RNA competes with 32s for hybridisation with DNA as do both 18s and 28s RNA with the 45s molecule. Purified 18s material, however, does not compete with 28s or 32s RNA. Comprehensive reviews of rpreRNA maturation have been published recently (Attardi and Amaldi, 1970; Maden, 1971; Burdon, 1971).

(b) Ribosomal Proteins.

At the commencement of this project, no methods had been developed which would permit complete fractionation of eukaryotic ribosomal subunit proteins, and so neither the absolute number of proteins on each subunit, nor their stoichiometry has been determined. Nevertheless, several investigations have indicated the approximate number of proteins to be expected on each eukaryotic subunit (see Table I (4)). The techniques used to obtain these results are subject to considerable error (King et al., 1971), but the report by Bickle and Traut (1971) is of interest in that they indicate the variability to be expected of their analysis by showing that their data for the ribosomal proteins of E.coli are within 10% of the known values for that organism. Moreover, their results for Euglena ribosomes coincide with those of Warner (1971) for yeast, and if it is assumed that all eukaryotic ribosomes contain approximately the same number of proteins, it can be estimated that 29 proteins of average molecular weight 25,700 daltons and 45 proteins of average molecular weight 28,700 daltons exist on the small and large subunits respectively, an increase over the total number and weight of prokaryotic (E.coli) ribosomal proteins of 35% and 106%. Also, the electrophoretic heterogeneity of eukaryotic ribosomal proteins (Warner, 1971; King et al., 1971; Ford, 1971; Bickle and Traut, 1971) indicate that, like the proteins of bacterial ribosomes, animal ribosomal proteins are very heterogeneous with respect to molecular weight.

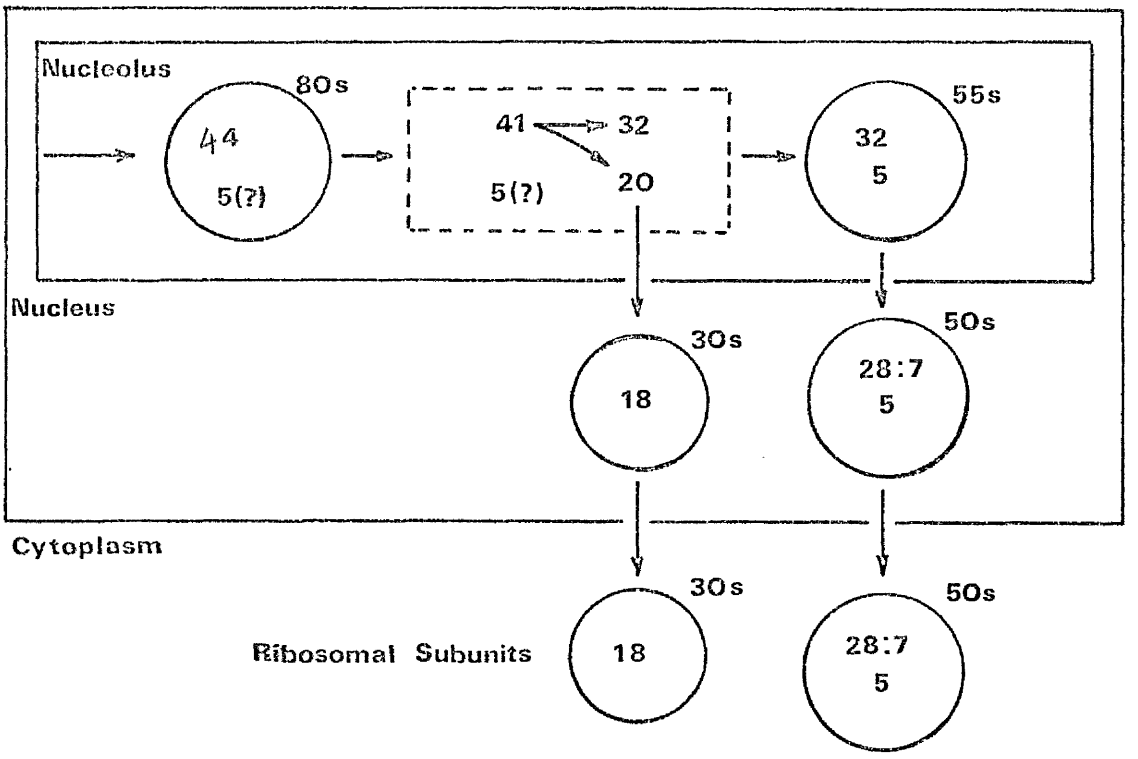


Fig. I (2)

Schematic representation of ribosome formation in eukaryotic cells (adapted from Maden, 1971). The numbers outside the particles represent the sedimentation coefficients of the particles while those inside indicate the sedimentation coefficients of the RNA species found in the particle concerned. The time sequence of particle maturation follows that described for rpreRNA maturation. Note that the 30s and 50s particles are found in the nucleoplasm only in very small amounts (30s $\ll$50s).

The amino acid composition of total ribosomal proteins (both eukaryotic and prokaryotic) is characterised by a comparatively high content of basic amino acids (arginine, lysine and histidine) which imparts a basic character to the proteins (Ts'o et al., 1958; Crampton and Petermann, 1959; Spahr, 1962; Mathias and Williamson, 1964; Westermann et al., 1971). Indeed, in contradistinction to prokaryotic ribosomes which have been shown to contain some acidic peptides (Waller, 1964; Low and Wool, 1967), mammalian ribosomal proteins appear to be entirely basic (Low and Wool, 1967). The ribosomal protein complex contains very little cysteine and may possess no disulphide bridges. The other sulphur-containing amino acid, methionine, is also present in small amounts (Westermann et al., 1971; Wu and Warner, unpublished).

(2) Mammalian Ribosome Assembly.

In this subsection, current knowledge of the mechanism of ribosome assembly is outlined by considering the types of ribonucleoprotein particles found in the nucleolus, the nucleoplasm, and the cytoplasm of mammalian cells. To this end, Fig. I (2) presents the concept of animal cell ribosome formation which existed at the outset of this project. The section is concluded by a description of recent data which imply the presence of considerable ribosomal protein "pools" in animal cells, and which suggest that mechanisms exist in these cells to ensure close coordination of synthesis of ribosomal components.

(a) Nucleolar ribonucleoprotein particles.

It has already been indicated that rpreRNA maturation occurs in the nucleolus. However, although ribosome-like objects were visualised in that organelle by electron microscopy some years ago (Bernhard and Granboulan, 1963), only relatively recently has it been possible to isolate such material from the cell nucleolus. Initial reports of successful isolation of these particles were made by Tamaoki and Mueller (1965) and by Tamaoki (1966). They described the purification, from HeLa cell nuclei of a heterogeneous group of ribonucleoprotein complexes sedimenting between 60 and 100s. However,

although the complexes were shown to contain high molecular weight RNA and some newly synthesised proteins, these proteins were not characterised, and so the significance of the complexes and their relationship to cytoplasmic ribosomal subunits was not established.

A more definitive study of nucleolar ribonucleoprotein particles was made by Warner and Soeiro (1967) who succeeded in extracting, from HeLa cell nucleoli purified by the method of Penman (1966), two such particle types with sedimentation coefficients in EDTA buffers of 80s and 55s. The larger, 80s particle was found to contain 45s RNA, some 32s,and (Soeiro, 1968) trace amounts of the 41s and 20s rpreRNA species described earlier (Fig. I (1)). 32s and 5s RNA were identified in the 55s nucleolar particle, suggesting a relationship between these particles and the mature 50s ribosomal subunit. This putative precursor-product relationship was confirmed by analysing the proteins of the 55s nucleolar particle and 50s ribosomal subunit on polyacrylamide gels. The two patterns showed striking correspondence, most of the proteins associated with the 28s RNA of the large ribosomal subunit being found also in the 55s particle. Moreover, "pulse-chase" experiments demonstrated a flow of protein radioactivity from the 55s to the 50s particle. The absence of one (or a small number of) 50s subunit protein(s) from the 55s nucleolar particle suggested the possibility of cytoplasmic addition of one or a few structural ribosomal proteins to the nascent 50s subunit. The existence of such a phenomenon had been indicated by earlier observations of Warner (1966). It should be noted that difficulty in isolating adequate quantities of protein from the 80s nucleolar particle precluded, at that time, complete investigation of its protein complement, although preliminary information suggested that this particle contained proteins destined for the 50s ribosomal subunit (Maden and Warner, unpublished).

Using a different isolation technique, Liau and Perry (1969) extracted, from L cell nucleoli, ribonucleoprotein complexes which, although of different sedimentation coefficient from those described by Warner and

Soeiro (1967), contained the same rpreRNA species as their HeLa cell counterpart. Furthermore, the buoyant densities of these nucleolar particles in caesium chloride were found to be significantly lower than the value characteristic of cytoplasmic ribosomal subunits, suggesting that the precursors have a relatively higher ratio of protein to RNA, and that ribosome maturation involves, in addition to modifications to the rpreRNA molecules, a progressive decrease in the proportion of particle-associated protein.

An interesting, yet completely unexplained phenomenon has been noted during labelling studies of nucleolar ribonucleoprotein particles. After a short "pulse" (e.g.10-20 minutes) with radioactive amino acids, tagged proteins appear rapidly and simultaneously on the 80s and 55s particles (Warner and Soeiro, 1967), although this short labelling period is of insufficient duration to permit de novo synthesis and maturation of a 45s rpreRNA molecule to its 32s product. The implications of these findings will be discussed later.

(b) Nucleoplasmic ribosomal precursor particles.

Maturation of nucleolar particles to ribosomes appears to be a nuclear event. Indeed, 30s and 50s ribonucleoprotein particles have been found in trace amounts in the nucleolus itself (Craig and Perry, 1970; Mirault et al., 1971). Moreover, Vaughan et al.(1967) have shown that it is possible to isolate, from nucleoplasm, ribonucleoprotein particles which, by a number of criteria, would seem to be nascent cytoplasmic ribosomes. The particles were found to contain RNA species identical to 28s and 18s rRNA, both in sedimentation characteristics and base composition (Vaughan et al., 1967); and 5s RNA (Knight and Darnell, 1967) was isolable from both 50s nuclear particles and cytoplasmic ribosomal subunits. Furthermore, kinetic data provided strong additional support for the notion that the nuclear 50s and 30s particles are cytoplasmic ribosomal subunit precursors (Vaughan et al., 1967). Finally, to refute the suggestion that these particles were non-specific aggregates of rRNA and protein (see Girard and Baltimore, 1966),

Vaughan et al. (1967) subjected the isolated material to centrifugation through high ionic strength gradients, a treatment known to inhibit non-specific RNA-protein interaction. The nuclear ribonucleoprotein complexes co-sedimented with their putative cytoplasmic counterpart. Unfortunately, however, because of the vanishingly small amounts of nuclear 30s and 50s particulate material, direct analysis of the proteins found on these two species was not possible.

The significance of the presence of these particles in the nucleoplasm is not completely clear. It is tempting to surmise that they may have been isolated as nascent ribosomes in transit from the nucleolus to their site of function in the cytoplasm, and the chemical and kinetic data mentioned above support this hypothesis. Indeed the relatively short half-life of such particles in the nucleoplasm, and the scarcity of 30s relative to 50s particles makes unlikely an alternative suggestion that these constitute permanent, functionally active nuclear ribosomes. Even their free existence in the nucleoplasm is questionable, since it is difficult to be certain to what extent the fractionation procedure induces the release of such ribonucleo-protein complexes from the nucleolus (Vesco and Penman, 1968).

(c) Newly formed ribosomes in the cytoplasm.

Considerable evidence now indicates that new ribosomes first appear in the cytoplasm as subunits (Girard et al., 1965; Perry 1965; Falvey and Staehelin, 1970) which contain mature 18s and 28s rRNA (Girard et al., 1965; Joklik and Becker, 1965). These nascent particles have a distinctly lower buoyant density in caesium chloride than "mature" large and small ribosomal subunits (Perry and Kelley, 1966a; Perry, 1967), but can be converted to particles whose density coincides with that of mature subunits by incubation with trypsin (Perry and Kelley, 1966b). Since the RNA components of "new" and "old" particles are identical, one interpretation of these experiments is that the newly formed species contain extra proteins which are responsible for their lower density. However, the amount of protein required to produce this density displacement (1.4×10^5 daltons) would be likely to increase the sedimentation coefficient of the"new" particles,

and Perry (1967) has suggested that the extra component associated with nascent particles may be very low density material such as membrane lipoproteins which become associated with the particles during their transit from the nucleolus. Alternatively, it may indeed be added protein which induces unfolding or opening of the particle structure, so fortuitously offsetting the effect of the increased particle mass on the sedimentation velocity (Perry, 1967). In this case, it would be reasonable to infer that the protein component of nascent ribosomes undergoes final modification in the cytoplasm.

A second interesting cytoplasmic modification to ribosomal subunits has already been mentioned. Warner and Soeiro (1967) found that most proteins of the mature 50s subunit were incorporated into that structure via its nucleolar precursors. One, or a small number of proteins, however, appears not to take part in de novo construction of the ribosome in the nucleolus, but becomes associated with the particle in the cytoplasm. In other words, there appears to be specific addition of protein to the 50s subunit during its cytoplasmic maturation. The findings of Perry (1967) and Warner and Soeiro (1967) are not mutually exclusive, but, taken together, imply that there is cytoplasmic modification of the protein complement of newly formed 50s subunits.

(d) Ribosomal protein "pools".

Ribosomal RNA maturation takes place concomitantly with assembly into ribonucleoprotein particles in the nucleolus (Warner and Soeiro, 1967; Liau and Perry, 1969), and present evidence implicates cytoplasmic ribosomes in the synthesis of ribosomal structural proteins (Speer and Zimmerman, 1968; Heady and McConkey, 1970; Kawashima et al., 1971; Craig and Perry, 1971). The proteins then appear to be transported to the nucleus (Kawashima et al., 1971).

A number of experiments have demonstrated that cells pretreated with cycloheximide to inhibit protein synthesis, continue to release appreciable quantities of 28s and 18s rRNA into the cytoplasm in the form of intact

ribosomal subunits (Warner et al., 1966; Maden and Vaughan, 1968; Willems et al., 1969; Craig and Perry, 1970). This finding would suggest that substantial "pools" of ribosomal proteins exist in the cells. Furthermore, the fact that much less new 18s than 28s rRNA appears in the cytoplasm after protein synthesis inhibition has prompted the suggestion that the 30s protein "pool" is smaller than its 50s counterpart (Maden and Vaughan, 1968; Maden, 1971). Other possible explanations of this finding do exist.

(e) Coordinated synthesis of ribosomal components.

Assembly of ribosomal components into a functional ribosome requires precise phasing. If synthesis of one component is inhibited, production of the other components is restricted and the superfluous material may ultimately be degraded, even if the inhibition is removed. This general principle appears to apply both to inhibition of rpreRNA and ribosomal protein synthesis. For example, cycloheximide, in addition to inhibiting ribosomal protein synthesis in animal cells, also reduces the rate of synthesis of 45s rpreRNA (Willems et al., 1969) and restricts its conversion to 32s RNA (Willems et al., 1969; Craig and Perry, 1970). Similarly, partial inhibition of protein synthesis by hypertonic medium (Pederson and Kumar, 1971) or valine starvation (Maden et al., 1969) is followed by reduction in the rate of 45s RNA processing in HeLa cells, and those particles produced in the presence of hypertonic medium are deficient in protein (Pederson and Kumar, 1971) (conversely, Craig and Perry (1970) found that cycloheximide modified particles have a higher protein content than normal, but suggest that this is the result of non-specific protein complexing to partially "naked" ribosomal precursor particles).

On the other hand, inhibition of rpreRNA synthesis by actinomycin D leads to inhibition of synthesis or accumulation of ribosomal proteins in the treated cells (Craig, 1971). A similar effect was seen after camptothecin-induced inhibition of rpreRNA processing (Wu et al., 1971). Ribosomal proteins, made in the presence of camptothecin do not accumulate in the nucleolus and are not subsequently used to form ribosomes, even if rRNA synthesis is allowed to resume.

I D Outline of present project

The above summary of present knowledge of mammalian ribosome assembly indicates that although the general mechanism of rpreRNA maturation is now well understood, little is known of the order of addition of ribosomal proteins to the nascent particles. In particular, the protein content of the 80s nucleolar particle, which contains RNA destined for both ribosomal subunits, has been poorly characterised. One possibility was that assembly of both subunits might commence on this particle. To test this possibility, it was important to select -

(a) a suitable source of mammalian ribosomal precursor particles and,

(b) a sensitive protein fractionating technique capable of resolving 50s from 30s proteins, and applicable to the study of the microgram amounts of protein which can be isolated from nucleolar 80s particles.

Cultured cells are a particularly good source of material for a study of the mechanism of ribosome assembly. They can be grown, under controlled conditions, to provide adequate amounts of ribosomal precursor material, whose composition may be modified by manipulation of the cells' environment. Also, standard procedures have been described (Penman 1966; Warner and Soeiro, 1967) for the isolation and purification of nucleolar particles, essentially free from cytoplasmic contamination. Thirdly, suitable radioactive isotopes are assimilated rapidly by the cells to provide a source of high specific activity ribosome precursors more economically than would be possible by the use of whole experimental animals.

The choice of fractionation procedure for the identification of ribosomal proteins found on precursor nucleolar particles was made by a process of elimination. A number of techniques were considered. Ion exchange column chromatography is a powerful tool in the fractionation of E.coli ribosomal proteins. However, its usefulness in the separation of eukaryotic ribosomal proteins was at first disappointing (Curry and Hersh, 1966), although, with this technique, Westermann et al. (1971) recently isolated, from total rat liver ribosomes, 31 proteins in relatively pure

form. Pilot studies using HeLa cell ribosomal proteins did not repeat this success, and the method was abandoned. Likewise, because eukaryotic proteins are strongly basic and possess similar isoelectric points, isoelectric focusing techniques were found to be unsuitable. Polyacrylamide gel electrophoresis remains the most powerful tool for the analysis of eukaryotic ribosomal proteins. The complexity of the protein mixture is such that separation of the proteins in one dimension gives inadequate resolution, but modification of the two dimensional electrophoresis system of Kaltschmidt and Wittmann (1970b) has permitted a more detailed analysis of the proteins of the eukaryotic ribosome (Welfle et al., 1971; Martini and Gould, 1971; Huyhn-van-Tan et al., 1971; Sherton and Wool, 1972). This method, however, will not provide an absolute estimate of the number of proteins in a complex mixture, but will act only as an adjunct to more definitive fractionation procedures (Mora et al., 1971). Moreover, the availability of large amounts of protein is a prerequisite of the two dimensional gel technique, and constitutesthe major impediment to the use of this method for the study of mammalian ribosome assembly.

The limited value of standard protein fractionation techniques for the purposes of this investigation necessitated the use of less commonly employed procedures, and it was found that modification of the peptide fingerprinting technique originally devised by Traut et al., (1967), and described earlier, resulted in a method which was suitable for the study of several aspects of mammalian ribosomal protein structure and formation. Essentially, the procedure involves in vivo labelling of the proteins of the ribosomes or their precursor particles with high specific activity L-[^{35}S] methionine or cystine, isolation of the particles, tryptic digestion of their proteins (without prior fractionation), separation of the peptides in two dimensions on paper, and location of radioactive peptides by autoradiography. Several advantages derive from this method:-

(i) The procedure is eminently suitable for the fractionation of microgram amounts of material, and provides reproducible results.

(ii) Laborious fractionation procedures are avoided.

(iii) Separation of the peptides occurs on the basis of the chemical structure of each protein in the complex mixture, and therefore is more specific than SDS gel electrophoresis which fractionates proteins purely on the grounds of their molecular weight (Weber and Osborn, 1969).

The fact that the procedure could not be used to determine the absolute number of proteins in a complex mixture did not limit its value in the investigation for which it was designed since only comparative data from each precursor particle and mature ribosomal subunit were required to make an assessment of the mechanism of ribosome assembly. For this purpose, the fingerprinting technique was almost ideal and provided useful information on mammalian ribosome structure and formation.

First, it showed that although most large ribosomal subunit proteins are isolable from the 80s nucleolar particle, only a few proteins from the small subunit could be identified as part of this structure.

Secondly, it furnished confirmatory evidence to support data on ribosome assembly which had been acquired by the use of entirely different techniques.

Thirdly, it made available additional information on the structure of cytoplasmic ribosomal subunits and their interaction with cytoplasmic proteins.

Finally, it proved to be a sensitive means of examining species variations of ribosomal protein structure.

The data on ribosome structure and formation derived by this technique, and supported by information acquired from other experimental sources has permitted the formulation of a tentative biosynthetic pathway for mammalian ribosomes.

MATERIALS AND METHODS.

Table II (1) Minimal Essential Medium (Glasgow Modification)

AMINO ACIDS	mg/l	VITAMINS	mg/l
L-Arginine HCl	42.1	D-Calcium pantothenate	2.0
L-Cystine	24.0	Choline Chloride	2.0
L-Glutamine	292.0	Folic Acid	2.0
L-Histidine HCl	19.2	i-Inositol	4.0
L-Isoleucine	52.5	Nicotinamide	2.0
L-Leucine	52.5	Pyridoxal HCl	2.0
L-Lysine HCl	73.1	Riboflavin	0.2
L-Methionine	14.9	Thiamin HCl	2.0
L-Phenylalanine	33.0		
L-Threonine	47.6		
L-Tryptophan	8.2		
L-Tyrosine	36.2		
L-Valine	46.9		

INORGANIC SALTS AND OTHER COMPONENTS

$CaCl_2.2H_2O$	264.0	NaCl	6400.0
Dextrose	4500.0	$NaH_2PO_4.2H_2O$	140.0
$Fe(NO_3)_3.9H_2O$	0.1	Phenol Red	17.0
KCl	400.0	$NaHCO_3$	2750.0
$MgSO_4.7H_2O$	200.0		

Penicillin 10^5 units

Streptomycin SO_4 $10^5 \mu g$

SECTION II - MATERIALS AND METHODS.

II A. Materials

(1) Biological

The following cell lines were used:-

Cell Line	Tissue Type and Source	Reference
HeLa (monolayer adapted)	Human Epithelium (Cervical carcinoma)	Gey et al., 1952
HeLa S3 (suspension culture)	Human Epithelium (Cervical carcinoma)	Puck et al., 1956
BHK-21/C13	Fibroblast (Baby Hamster kidney)	Macpherson and Stoker, 1962
L929	Fibroblast (Mouse)	Sanford et al., 1948.

Primary cultures of chick embryo cells were prepared as in METHODS.

Tissue Culture Media

Eagle's Minimal Essential Medium (MEM)

Cell lines and primary cultures grown as monolayers were propagated in Eagle's MEM (Glasgow Modification - Busby, House and MacDonald, 1964) to which had been added 0.2µg of n-butyl p-hydroxy benzoate (an antimycotic agent), calf serum to 10% (v/v), phenol red to 0.002% (w/v), penicillin (100 units/ml) and streptomycin sulphate (100µg/ml). This medium is designated "EC 10" in this report. Suspension cultures were maintained in Eagle's MEM (Joklik modification) containing 10% calf serum (Joklik modified EC 10), purchased from Schwarz Mann, Orangeburg, N.Y.
The constituents of Eagle's MEM (Glasgow modification) are shown in Table II (1). Methionine deficient amino acid concentrate (50x) was purchased from Biocult Laboratories Ltd., Paisley, Scotland, or was prepared according to Eagle's (1959) formulation.

Balanced Salt Solution (BSS : Earle 1943)

Contained 0.116M NaCl, 5.4mM KCl, 1mM $MgSO_4$, 1mM NaH_2PO_4, 1.8mM $CaCl_2$ and 0.002% (w/v) phenol red. The pH of this solution was adjusted to 7.0 with 8.4% (w/v) $NaHCO_3$.

Phosphate Buffered Saline (PBS : Dulbecco and Vogt, 1954)

Consisted of 0.17M NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 2.4mM KH_2PO_4, 0.49mM $MgCl_2.6H_2O$ and 0.68mM $CaCl_2.2H_2O$. The pH was adjusted to 7.4 and 0.002% phenol red added.

Phosphate Buffered Saline (A)

Calcium- and magnesium-free PBS. As for PBS, but without addition of $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$.

Versene

0.6mM EDTA in PBS

Trypsin/citrate

0.25% (w/v) trypsin, 10.5mM NaCl, 1.0mM Sodium citrate and 0.002% phenol red, adjusted to pH7.8 with NaOH.

Versene/trypsin

Versene 4 parts, trypsin/citrate 1 part(v/v)

(2) Chemical

(a) General

Tween 80, acrylamide, N,N' methylenediacrylamide, adenosine, guanosine and polyethylene glycol (mol. wt. 20,000 and 6,000) were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

N,N,N',N' - tetramethylenediamine (TEMED), ammonium persulphate, disodium ethylene diamine tetra acetate (EDTA), thioglycollic acid and 30% (w/v) hydrogen peroxide were purchased from BDH Biochemicals Ltd., Poole, Dorset, while caesium chloride was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. Dithiothreitol (Cleland's Reagent) was purchased from Calbiochem Ltd., London.

(b) Radiochemicals

Were purchased from the Radiochemical Centre, Amersham, Bucks.,and included [5-^{3}H]uridine (5Ci/mmol); [2-^{14}C]uridine (60mCi/mmol); L-[4,5-^{3}H]leucine

(17-19Ci/mmol); L-[U-^{14}C]leucine (312-331mCi/mmol); L-[U-^{14}C]valine (260mCi/mmol); L-[Me-^{3}H]methionine (8.3Ci/mmol); L-[Me-^{14}C]methionine (56mCi/mmol); L-[^{35}S]methionine (500mCi/mmol-19Ci/mmol); L-[^{35}S]cystine (200mCi/mmol); L-[2,3-^{3}H]phenylalanine (10mCi/mmol); L-[4,5-^{3}H]lysine (250mCi/mmol).

L-[^{35}S]methionine was also obtained more economically by utilising the biosynthetic procedure of Bretscher and Smith (1972):-

50ml of sterile M9 medium (40mM Na_2HPO_4, 17mM KH_2PO_4, 10mM NaCl, 20mM NH_4Cl) supplemented with 1.0mM $MgCl_2$, 0.05mM $MgSO_4$ and 1% glucose were inoculated in a Drechsel flask with one drop of stock E.coli B culture, and aerated gently for 18 hours after addition of 20mCi[^{35}S]sulphate (8.9mCi/mmol; 0.1 mg/ml). The effluent air was passed through a 1.0M NaOH solution to trap any H_2S which might be produced. Towards the end of incubation, the uptake of radioactivity was assayed by measuring total, and membrane filter-retained radioactivity (equivalent to radioactivity in bacteria) in small aliquots removed from the culture. From these values, uptake of label was calculated to be approximately 40% of the total radioactivity added.

At the end of the incubation period, the bacteria were precipitated by centrifugation at 25,000g for 30 minutes, and the pellet resuspended in 0.25ml H_2O and transferred to a pyrex tube. The centrifuge tube was washed, first with 0.25ml H_2O, then with 0.5ml 12M HCl, and the washings added to the pyrex tube. 0.1ml thioglycollic acid was added to the mixture which was frozen, sealed off in vacuo, and hydrolysed at 105°C for 18 hours.

The hydrolysate was lyophilised twice, dissolved in 0.1M βmercaptoethanol, and streaked onto Whatman 3MM paper. Fractionation was by descending chromatography for 16 hours using the upper phase of a mixture of n-butanol:acetic acid:water (4:1:5). In this system, L-methionine is cleanly separated from L-cysteine and L-cystine (respective Rf. values

0.55, 0.35, 0.10).

The L-[^{35}S]methionine was located by autoradiography (approximately 20 seconds exposure of Kodirex sheet film) and eluted from the paper with 5mMβ-mercaptoethanol. The pH was adjusted to 8.0 with KOH, and the material stored at -20°C.

(c) Drugs and Inhibitors

Actinomycin D and L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) were purchased from Calbiochem Ltd., London.

Toyocamycin (4-amino-5-cyano-7-β-D-ribofuranosyl-pyrrolo (2,3-d) pyrimidine - Fig. III (26)) was a gift to Dr. R.H. Burdon of this department from Dr. G. Acs, Institute for Muscle Disease, New York.

(d) Enzymes

Ribonuclease A (bovine pancreatic, protease-free; EC2.7.7.16.) was purchased from the Sigma Chemical Co., Ltd., London; Ribonuclease T_1 (extracted from Aspergillus oryzae) from Calbiochem Ltd., London; and electrophoretically purified deoxyribonuclease(EC3.1.4.5.)and three times recrystallised trypsin (EC3.4.4.4.) from Worthington Biochemical Corp., New Jersey.

(e) Chromatography/Electrophoresis

Whatman 3MM paper (46x57cm) and DEAE 81 paper rolls (46cm x 50m) were purchased from H. Reeve-Angel and Co. Ltd., London.

Cellulose acetate electrophoresis strips (25cm x 95cm) were obtained from Oxoid Ltd., London.

(f) Scintillation Spectrometry

2.5cm diameter cellulose acetate filters (o.45μ pore size) were purchased from Sartorius-Membrane filter GMBH, 34, Gottingen, West Germany.

2,5 diphenyloxazole was bought from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and toluene and 2-methoxyethanol from BDH Biochemicals Ltd., Poole, Dorset.

Toluene based (non water miscible) scintillation fluid was prepared by

dissolving 5gm of 2,5 diphenyloxazole in 1 litre of toluene.

Water miscible scintillant was obtained by mixing toluene/2,5 diphenyloxazole with 2-methoxyethanol in the ratio 1/1(v/v).

(g) Miscellaneous

14"x17" Kodirex sheet film, D19 developer and Kodafix solution were purchased from Kodak Ltd., London.

II B. Methods.

(1) General Cell Culture Techniques

(a) Monolayer Cell Lines

HeLa, BHK-21/C13 and L929 cell lines were cultured routinely as monolayers in rotating 80oz.winchester bottles according to the method of House and Wildy (1965). $25x10^6$ cells were seeded into 200ml of EC10 and incubated at 37°C for 2-3 days in a 5% CO_2 atmosphere until the cell density had reached approximately 10^8/bottle.

For serial passaging, the cells were removed from the glass by trypsin/versene (see later), suspended in EC10 and dispensed in $25x10^6$ cell aliquots into sterile 80oz.bottles containing 200ml of EC10. Cell cultures were maintained by the staff of the Wellcome Cell Culture Unit of this department.

(b) HeLa S3 Suspension Cells

were maintained in spinner flasks (Bellco Glass Inc., Vineland, N.J.) at 37°C. Cell density was kept between $30-60x10^4$/ml by daily dilution of the cell stock with Joklik modified EC10.

(c) Primary Chick Cultures

10 day fertilised eggs were cracked open under sterile conditions, and the embryo removed and decapitated. The carcase was washed thrice in PBS(A), minced finely with scissors, and the minced tissue washed repeatedly in PBS(A) until cleared of red blood corpuscles. The PBS(A) was removed with a pasteur pipette and the tissue fragments incubated, with agitation in 10ml trypsin/citrate for 15 minutes at 37°C. The fragments were then allowed to settle, the cell suspension removed, and the trypsin neutralised with calf serum. The procedure was repeated on the pellet as required, until disaggregation was complete.

The cell suspensions were pooled, the cells pelleted by centrifugation (800g, 5 minutes), washed in PBS, and recentrifuged. The washed pellet was dispersed in EC10, and large particulate material allowed to precipitate. Aliquots of $200x10^6$ cells were dispensed into 200ml EC10

in 8Ooz.bottles. After overnight incubation at 37°C, the medium, containing debris, was decanted and replaced by fresh EC10. The cells were used within 48 hours. I acknowledge the expert technical assistance of Miss Margaret Nielson in the preparation of primary chick cultures.

(d) Contamination Checks

All sterile media and passaged cells were checked regularly for bacterial, fungal or PPLO infection as follows:-

Bacterial contamination

Aliquots were grown on blood agar plates and brain-heart infusion broth at 37°C. Results were considered negative if no growth was seen within 7 days.

Fungal contamination

Aliquots were added to Sabouraud's medium and grown at 32°C. Again, no growth after 7 days was assumed to indicate the absence of mycotic contamination.

PPLO infection

PPLO agar plates were seeded with passaged cells by piercing the agar surface with a charged pasteur pipette. The plates were grown in an atmosphere of 5% CO_2 in N_2 at 37°C. To test the efficacy of the method, PPLO infected cultures were prepared as controls. After 7 days inoculation, the plates were examined microscopically for the characteristic "fried egg" appearance of PPLO colonies.

(2) Cell Labelling and Harvesting

(a) Labelling of cells with radioactive amino acids

When cells are grown in medium completely or partly deficient in one or more amino acids, the rate of protein synthesis is reduced (Eagle et al., 1959; Shields and Korner, 1970). Moreover, when HeLa cells are deprived of the essential amino acid valine, the rate of formation of new ribosomes declines (Maden et al., 1969), and in methionine-deficient media, production of completed ribosomes is arrested (Vaughan et al., 1967).

It was therefore essential in this study of the structural proteins of eukaryotic ribosomes that amino acid concentrations in the medium were not limiting to cell growth. In most experiments, cells were labelled with methionine. Fig. II (1) shows that, in methionine deficient media, the rate of incorporation of L-[^{35}S]methionine into HeLa cells is $2\times10^{-4}\mu$ moles/hour/10^6cells. From this result, it can be calculated that 4×10^8 cells will incorporate 0.05μ moles of L-[^{35}S]methionine in approximately 40 minutes (a subsequent experiment whose result is not shown indicated that, under these conditions of cell density and methionine concentration, incorporation was linear for 45 minutes). All subsequent experiments involving the incubation of approximately 4×10^8 cells with 0.05μ moles L-[^{35}S]methionine were restricted to labelling times of 30-45 minutes to prevent the appearance of methionine starvation effects. These incubation conditions, indeed, were found to be ideal for labelling nucleolar particle proteins to high specific activity, or for kinetic analyses of the flow of protein radioactivity through maturing ribonucleoprotein particles.

Similarly, in studies requiring equilibrium labelling of cell proteins with L-[^{35}S]methionine over 68-72 hour periods (e.g. investigations of stable structural ribosomal proteins), incubation was performed in normal EC10; when the incubation period was 18-24 hours, EC10 containing 1/5th normal methionine concentration was used. In neither case should more than 2/3rds of the added methionine be taken up into the cells.

(b) Harvesting of monolayer cells

Cells were harvested from glass in one of two ways.

(i) Mechanical scraper

Growth medium was decanted and the cell sheet rinsed twice with ice cold BSS. The cells were removed from the glass into 50ml of freshly added BSS by means of a rubber scraper, transferred to a 50ml centrifuge tube, pelleted by centrifugation at 800g for 2 minutes (+4°C), and washed twice

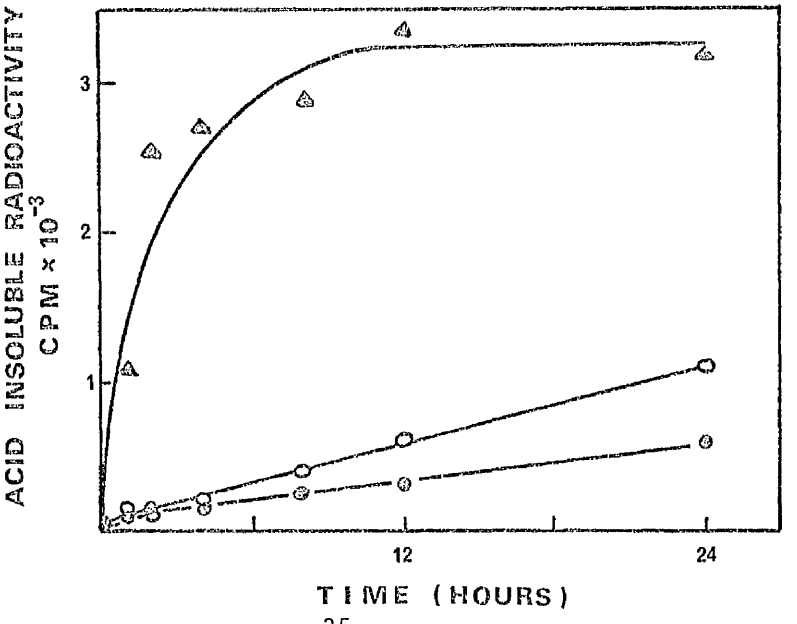


Fig. II (1) Incorporation of L-[^{35}S]methionine into HeLa cells

Three flasks were prepared containing

A: 50ml of Joklik modified EC 10 with no methionine
B: 50ml of Joklik modified EC 10 with 0.02mM methionine
C: 50ml of Joklik modified EC 10 with 0.1mM methionine

The flasks, preincubated at 37°C were each inoculated with 12.5 x 10^6 HeLa S3 cells, followed by 10μCi L-[^{35}S]methionine (0.02μ moles). At intervals, 0.01ml aliquots were sampled and made 10% with TCA. Acid insoluble material was collected on membrane filters and radioactivity measured in a Nuclear Chicago low background gas flow counter.

Flask (A), containing 0.02μ moles L-methionine —Δ—Δ—
Flask (B), containing 1.2 μ moles L-methionine —o—o—
Flask (C), containing 5.2 μ moles L-methionine —ø—ø—

In 8 hours, the cells in flask (A) utilised 0.02μ moles of L-methionine. i.e. incorporation rate is 2 x 10^{-4}μ moles/hour/10^6 cells.

in 50ml cold BSS with intermediate centrifugation.

(ii) Trypsin/Versene

After removal of growth medium, the cell sheet was washed twice with 50ml aliquots of BSS, and 25ml trypsin/versene at 37°C added to dislodge cells from the glass. The cell suspension was decanted immediately into a chilled (0°C) 50ml centrifuge tube containing 2.5ml serum to neutralise tryptic activity. After pelleting at 800g for 5 minutes (+4°C), the cells were washed twice with ice cold BSS.

(3) Cell Fractionation

(a) Ribosome preparation

Ribosomes were isolated according to the method of Warner (1966). All steps were carried out at +4°C. The cell pellet, after removal from glass and washing with BSS as described above, was carefully drained of residual BSS and suspended in hypotonic RSB (0.01M NaCl; 0.0015M $MgCl_2$; 0.01M Tris/HClpH7.4). 4.0ml of RSB were added to 10^8 cells, and the cells allowed to swell by standing in ice for 10 minutes. Disruption was completed by homogenising with 25 strokes of a stainless steel Dounce homogeniser (clearance of 0.003" gave maximal cell rupture with minimal nuclear damage), and nuclei and unbroken cells were pelleted by centrifugation (1500g,5 minutes) and used for nucleolar purification if required.

The supernatant, containing ribosomes, was centrifuged at 10,000g for 10 minutes to precipitate mitochondria, and to the remaining cytoplasm was added tween 80 to 0.5%,sodium deoxycholate to 0.5% and, finally, $MgCl_2$ to 0.07M (Takanami, 1960; Attardi and Smith, 1962), with thorough mixing between additions. After 45 minutes, the bulk of the ribosomes had aggregated and could be collected by centrifugation at 10,000g for 10 minutes. The ribosomes were then dissociated into subunits and analysed on sucrose gradients in one of two ways:-

(i) EDTA dissociation

The ribosome pellet was suspended in 1.0ml NEB (0.01M NaCl; 0.01M EDTA; 0.01M Tris/HClpH 7.4), first by gentle agitation with a glass rod, then by homogenisation (10 strokes of a loose-fitting glass homogeniser), and the subunits separated in 15-30% (w/w) sucrose gradients in NEB by centrifugation (+4°C) for 17 hours at 80,000g.

(ii) 0.85M KCl dissociation (Martin and Wool, 1968)

Alternatively, the ribosomes were suspended as above, but in 1.0ml of 0.85M KCl buffer (0.85M KCl; 15mM $MgCl_2$; 50mM Tris/HClpH 7.8), and separated into subunits in 15-30% (w/w) sucrose gradients in 0.85M KCl buffer as described above.

Typical absorbance profiles of (i) and (ii) are shown in Figs. III (2) and (3) respectively.

The sedimentation coefficient of EDTA dissociated large and small subunits is 49.9s and 28.6s whereas that of KCl dissociated subunits is 59.1s and 40.9s respectively (Hamilton et al., 1971). For simplicity, these shall be termed 50s, 30s, 60s and 40s subunits.

(b) Isolation of nucleolar particles and nucleolar RNA

Warner and Soeiro's(1967) modification of Penman's (1966) method of nucleolar purification was adopted to prepare nucleolar particles. All manipulations were performed at 0°C unless otherwise specified.

The nuclear pellet, derived by RSB lysis and Dounce homogenisation, was washed with 4.0ml of RSB, then suspended in 4.0ml RSB to which was added 0.6ml of a solution containing 6.7% tween 80 and 3.3% Na deoxycholate. The suspension was mixed briefly on a vortex mixer and the nuclei pelleted by centrifugation at 800g for 5 minutes. This treatment removes residual cytoplasmic contamination and unruptured cells (Penman, 1966). Residual detergent was removed from the nuclear pellet by rinsing its surface with 1.0ml of RSB. This facilitated the subsequent action of DNase (Pederson and Kumar, 1971). The gelatinous pellet was suspended in 2.5ml of HSB

(0.5M NaCl; 0.05M $MgCl_2$; 0.01M Tris/HClpH 7.4), 100µg of DNase added, and the mixture incubated at 37°C with vigorous pipetting, using a broken off wide bore pasteur pipette, until all DNA clumps were dispersed and the viscosity of the solution was reduced considerably (approximately 1 minute incubation was sufficient).

It appeared that different batches of DNase contained variable amounts of contaminant RNase. For this reason, it was important to minimise both the incubation times and the concentrations of DNase employed in the nuclear fractionation procedure. The crude nucleoli were collected by centrifugation at 10,000g for 10 minutes. The nucleolar pellet was then extracted in one of two ways to prepare -

(i) Nucleolar particles

(ii) Nucleolar RNA

(i) Nucleolar particles

The nucleolar pellet was dispersed in 1.0ml of NEC (0.01M dithiothreitol in NEB), first by gentle agitation with a glass rod, then by homogenisation (10 strokes of a loose-fitting glass homogeniser). The nucleolar particles were extracted by stirring gently for five minutes at room temperature. This extraction step is critical. Underextraction of pelleted nucleoli results in severely reduced yields of nucleolar particles. Conversely, extraction for periods longer than five minutes, especially at elevated temperatures (e.g. 37°C) was found to cause an unacceptable degree of 80s and 55s particle degradation, possibly due to the presence of contaminant RNase. Recentrifugation of the extracted nucleolar material at +4°C for 10 minutes (10,000g) precipitated nucleolar debris, and the supernatant, containing the nucleolar particles, was layered on a 15-30% (w/w) sucrose gradient in NEB. Centrifugation at +4°C for 16 hours (70,000g) separated the particles. A typical absorbance profile, showing 80s and 55s peaks, is seen in Fig. III (5).

(ii) Nucleolar RNA

Nucleolar RNA was prepared by dissolving the nucleolar pellet in 1ml LETS (0.1M LiCl; 0.001M EDTA; 0.2% SDS; 0.01M Tris/HCl,pH 7.4) and separating the rpreRNA species (45s and 32s) by centrifugation in 15-30% (w/w) sucrose gradients in LETS for 16 hours at 20°C (62,000g).

After centrifugation, all gradients were collected into approximately 30 equal fractions, while the A260mμ was monitored continuously by a Gilford 240 recording spectrophotometer.

(4) Protein and RNA fingerprinting techniques

(a) Preparation of TPCK-trypsin

Investigations of protein structure have made extensive use of trypsin to obtain specific cleavage of peptide chains at the carboxyl groups of lysine and arginine. Unfortunately for such studies, it has been noted that many samples of crystalline trypsin display appreciable chymotryptic activity. Schoellman and Shaw (1963) have shown that chymotrypsin is labelled specifically at the active centre by L(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK), whereas trypsin is relatively unaffected by it. Kostka and Carpenter (1964) devised a method of treating trypsin samples with TPCK and showed that the treated enzyme was freed of chymotryptic activity. TPCK-trypsin was prepared by the method of Kostka and Carpenter (1964), the essentials of which are as follows:-
1gm trypsin (Worthington, 3x recrystallised) was dissolved in 330ml 0.001M $CaCl_2$ and 284.1mg of TPCK in 7.5ml anhydrous methanol were added. The reaction mixture, which became turbid owing to precipitation of part of the inhibitor, was titrated to pH 7.0 with 0.5N NaOH and maintained at this pH by regular addition of alkali as required for 304 minutes. The reaction mixture was then brought to pH 3 with 1N HCl, excess inhibitor removed by filtration, and the filtrate dialysed at +4°C against 20 litres

of water at pH 3. The dialysed solution was lyophilised and the TPCK-trypsin stored at -20°C. The yield of TPCK-trypsin was 88%.

(b) Performate oxidation of proteins.

Oxidation of many proteins renders them more susceptible to the action of trypsin, and the cleavage of S-S bonds prior to enzymic hydrolysis is likely to result in the production of simpler peptides than would be formed were the disulphide bridges left intact (Hirs, 1956). Performic acid reacts predominantly with cystine, tryptophan, and methionine, but under certain conditions, oxidises other amino acids also (Toennies and Homiller, 1942). In particular, tyrosine is converted to monochloro-tyrosine by the action of chlorine liberated when hydrogen peroxide reacts with chloride (Thompson, 1954). However, by using the method of Hirs (1956), chlorotyrosine formation does not occur, and cystine, tryptophan and methionine are oxidised reproducibly in more than 90% yield. The method involved pre-preparation of performic acid by adding 0.5ml of 30% H_2O_2 to 9.5ml of 99% formic acid and allowing to stand for two hours at room temperature in a stoppered flask. The performic acid was then cooled to -10°C. The proteins to be oxidised were also pre-cooled to -10°C in a glass tube to which was added 0.05ml 99% formic acid, 0.01ml anhydrous methanol (to prevent freezing), and 0.1ml pre-cooled performic acid. The reactants were maintained at -10°C for 2.5 hours, 0.5ml distilled water was added, and the mixture lyophilised. A second lyophilisation completed volatilisation of the performic acid. The amounts of reactants used ensured oxidation of all susceptible amino acid residues by providing an excess of performic acid.

(c) Protein Fingerprinting (Shepherd and Maden, 1971; 1972)

The method used was a modification of that described by Traut et al. (1967). L-[^{35}S](methionine or cystine)-labelled ribosomal subunits or nucleolar particles prepared by the methods described were isolated from sucrose

gradients by pooling appropriate peak fractions. The ribonucleoprotein content of the pooled fractions was calculated from the absorbance at 260mμ measured on a Gilford 240 spectrophotometer ($E^{1\%}_{1cm}$ = 112: Rich, 1967), and the radioactivity determined by counting a small aliquot on a Nuclear Chicago gas flow counter. The pooled peak fractions were treated overnight with pancreatic ribonuclease (Sigma), 25μg/ml, at room temperature, with simultaneous dialysis against 2 x 5 litres of water containing thymol to inhibit bacterial growth. As RNA digestion proceeded, the released proteins formed a white flocculent precipitate, and preliminary experiments (Fig. II (2)) indicated that digestion was completed within one hour.

The protein suspension was lyophilised, performate oxidised as above, relyophilised twice to remove performic acid, and digested for 6 hours at 37°C with TPCK-trypsin in 0.1M NH_4HCO_3, pH 8.5 (Enzyme: substrate ratio = 1:40). Fig. II (3) shows that with this enzyme:substrate ratio, approximately 80% tryptic digestion of ribosomal proteins is completed within 6 hours. Because of the increasing danger of non-specific cleavages on prolonged incubation, digestion was usually terminated after this period. The digest was lyophilised three times to remove NH_4HCO_3 and the peptides separated by electrophoresis (2 hours, 2.5 KV, 1.5% pyridine acetate buffer, pH 4.7) in a Miles high voltage flat bed apparatus (Miles Hivolt Ltd., Shoreham, Sussex), followed by descending chromatography (16 hours, n-butanol:acetic acid:H_2O = 12:3:5). The radioactive peptides were located by autoradiography.

(d) RNA Fingerprinting

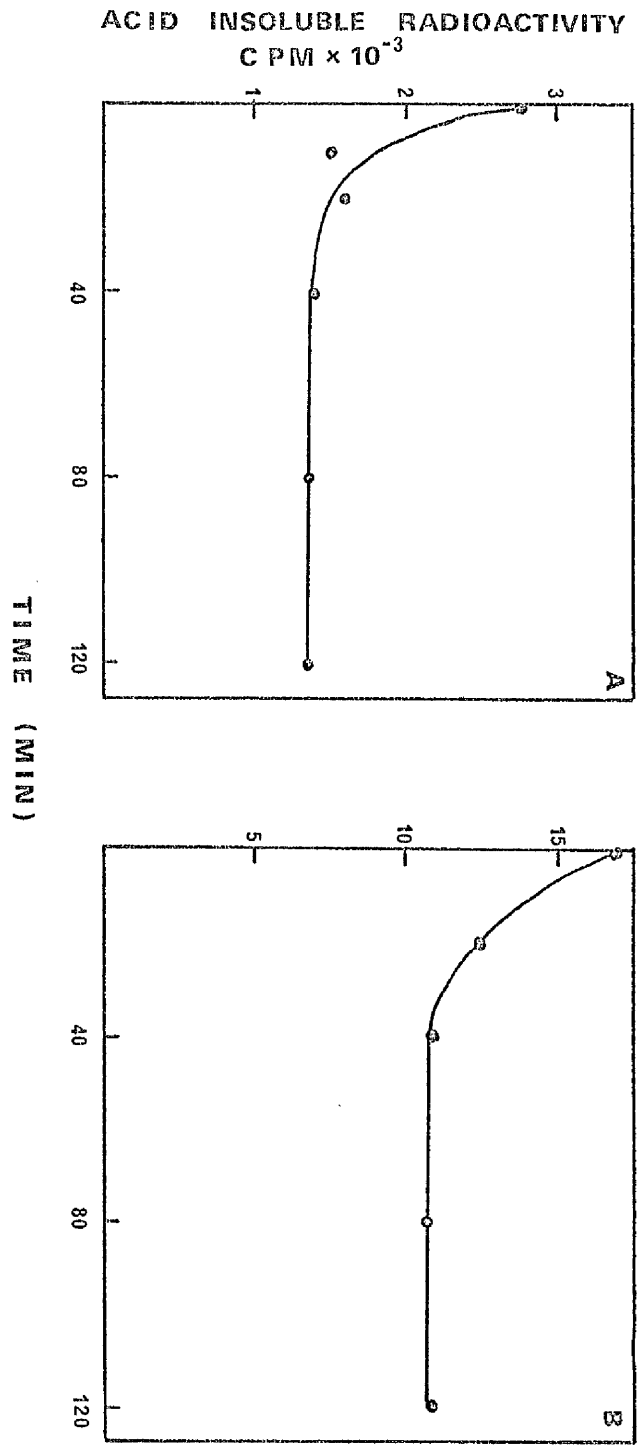
RNA fingerprinting was performed by the method of Sanger et al. (1965). 80s and 55s nucleolar particles, prelabelled with L-[methyl-^{14}C]methionine in the presence of adenosine/guanosine (each at 2×10^{-5}M) and 10mM sodium formate to inhibit purine ring labelling, were extracted and separated on sucrose gradients as described. The peak fractions were pooled separately and the particles precipitated overnight with 2.5 volumes of

Fig. II (2) Ribonuclease digestion of HeLa ribosome subunits

25×10^6 HeLa monolayer cells were incubated for 18 hours at 37°C in 200ml EC 10 containing 20μCi L-[methyl-^{14}C]methionine (final specific activity=1mCi/mmol). The cells were harvested by trypsinisation, ribosomes prepared, dissociated with EDTA and separated into subunits in a 36ml 15-30% sucrose gradient in NEB (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Subunit peak fractions were pooled. 1.0ml aliquots of each subunit type were incubated, for the times shown, at 37°C in the presence of 25μg RNase (sigma). Enzymic hydrolysis was terminated by addition of TCA to 10%, acid insoluble material collected on membrane filters and radioactivity measured in a Nuclear Chicago low background gas flow counter.

Panel A: 30s ribosomal subunits

Panel B: 50s ribosomal subunits


ACID INSOLUBLE RADIOACTIVITY
CPM x 10^-3
1
2
3
40
80
120
A
TIME (MIN)
5
10
15
40
80
120
B

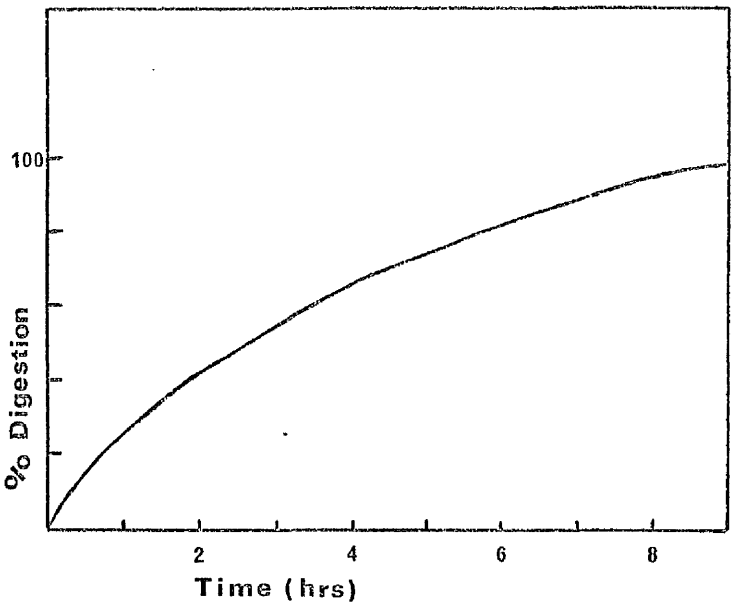


Fig. II (3) Rate of ribosomal protein digestion by TPCK-trypsin

Rat liver ribosomes were prepared by the method of Wettstein et al. (1963) and their proteins extracted with LiCl/urea after Leboy et al. (1964). The proteins were dialysed against distilled water to remove salt and urea, and were then lyophilised. 5mg of lyophilised protein were suspended in 3.0ml distilled water and equilibrated to pH 8.0 with 0.01 NaOH in a T.T.T.1. automatic titrator (Radiometer, Copenhagen). 0.125mg of TPCK-trypsin (enzyme/substrate ratio = 1/40) were added to the protein suspension and tryptic hydrolysis of the proteins at pH 8.0 followed to completion using the automatic titration facility of the T.T.T.1. titrator. The results are expressed as percentage digestion completed against time.

absolute ethanol at -10°C. The particles were collected by centrifugation at 5,000g for 30 minutes (-10°C), resuspended in 0.5ml 0.05M NaCl, and extracted twice with cold phenol. The aqueous phase, containing the RNA was cleared of phenol by extracting twice with ether, and residual ether removed by evaporation in a stream of air. NaCl was added to a final concentration of 0.15M and the RNA reprecipitated with 2.5 volumes of ethanol.

After pelleting by centrifugation as above, the RNA was dissolved in 1.0ml H_2O, an aliquot taken for radioactivity determination on a Nuclear Chicago gas flow counter, and the RNA concentration determined by measuring the absorbance at 260mμ in a Gilford 240 spectrophotometer ($E_{1cm}^{1mg/ml} = 22$: Gould and Simkins, 1969). The RNA content of the sample was adjusted to 60μg by addition of cold HeLa 28s RNA (20-100μg is satisfactory), the sample lyophilised and redissolved in 10μl of 0.01M Tris, 0.001M EDTA pH 7.5 containing T_1' ribonuclease to give an enzyme:RNA ratio of 1:20. After digestion in the tip of a drawn-out capillary at 37°C for 30 minutes in a humidified incubator, the products were separated by electrophoresis using cellulose acetate strips at pH 3.5 in the first dimension and DEAE paper with 7% formic acid in the second dimension (Sanger et al., 1965). The radioactive spots were located by autoradiography.

(5) Autoradiography

Kodirex 14" x 17" sheet film was used to locate radioactive spots. It was essential that fingerprints were dried thoroughly before autoradiography to prevent darkening of the photographic emulsion by the solvents. 150×10^3 DPM of applied material gave satisfactory autoradiographs within three weeks, while, if radioactivity was very low (e.g.10,000 DPM), up to 6 months' exposure was required. The autoradiographs were developed in Kodak D19 developer and fixed in Kodafix.

(6) CsCl buoyant density and acrylamide gel electrophoresis techniques

(a) CsCl centrifugation of ribonucleoprotein particles.

Radioactive cytoplasmic ribosomal subunits or nucleolar particles were separated, as described, on sucrose gradients and pooled peak fractions made 8% with glutaraldehyde, pH 7.4 (Baltimore and Huang, 1968). The fixed particles were layered on 5ml 33-55% (w/w) CsCl gradients in RSB and centrifuged to equilibrium (16 hours, +4°C, 100,000g). The gradients were collected into approximately 30 split fractions, and the refractive index of 1/5th of each fraction determined in an Abbe refractometer and related to densities (Vinograd and Hearst, 1962). The remainder of each fraction was made 10% with respect to TCA, acid insoluble radioactive material collected on membrane filters, and assayed by liquid scintillation spectrometry in a Packard Tricarb spectrometer.

(b) Protein preparation for acrylamide gel electrophoresis

Ribosomal subunits, isolated on sucrose gradients, were ethanol-precipitated from the pooled peak fractions, pelleted by centrifugation (-10°C) at 5000g for 30 minutes, and RNA and protein dissociated by addition of a small aliquot (0.1-0.2ml) of gel sample buffer (see below). 100µg, or less, of ribosomal proteins were separated on acrylamide gels as described below.

(c) Acrylamide gel electrophoresis

The method of King et al. (1971), a modification of methods previously described (Shapiro et al., 1967; Dunker and Rueckert, 1969; Weber and Osborn, 1969; Shapiro and Maizel 1970) was adopted.

10cm gels of 10% polyacrylamide were prepared immediately before use in 15cm x 0.5cm glass tubes. The gels contained 10% acrylamide, 0.35% N,N' methylene diacrylamide, 0.1% sodium dodecyl sulphate, 0.1M sodium phosphate buffer pH 7.1, 6M urea, 0.1% TEMED and 0.05% ammonium persulphate. Before polymerisation, the gel surface was overlayered with water which was replaced by reservoir buffer (0.1% SDS, 0.005M thioglycollic acid, 0.1M sodium phosphate buffer, pH 7.1) when polymerisation was complete.

The ribosomal protein, dissolved in 50-100µl of sample buffer (6M urea; 1% SDS; 1%β-mercaptoethanol; 0.01M sodium phosphate buffer, pH 7.1) was layered over the gel surface and electrophoresis continued for 6.5 hours at 10mA/gel (approx. 80 volts). After separation was complete, the gels were stained overnight in 10% acetic acid, 50% methanol and 0.1% coomassie blue (G.T. Gurr, Romford, Essex) in water, and destained by diffusion in 10% acetic acid, 10% methanol.

(d) Acrylamide gel processing

(i) Densitometry

Stained gels were placed in a 10cm glass cuvette and scanned at 599mµ using a Gilford 240 spectrophotometer with gel scanning attachment.

(ii) Slicing and radioactivity determination

After scanning, radioactive gels were fractionated into 1mm segments using a Mickle gel slicer (Mickle Laboratory Engineering Co., Gomshill, Surrey). Slices containing prominently stained peaks were noted to allow location of the radioactive profile on the densitometric trace. The gel slices were dried in scintillation vials at 80°C for 1 hour, 0.3ml 30% (w/v) H_2O_2 was added to each vial, the vials capped, and incubation continued at 60°C until all gel slices had dissolved. The coomassie blue coloration in peak slices disappeared during this procedure. To the dissolved gels were added 10ml of water miscible scintillant (see MATERIALS) and the samples counted in a Packard Tricarb liquid scintillation spectrometer. This method is a modification of Tishler and Epstein (1968).

RESULTS.

SECTION III A - General characterisation of ribosomal subunits and nucleolar particles.

The preliminary characterisation experiments described in this section were performed to define the optimal conditions for isolation and purification of ribosomal subunits and their precursor nucleolar particles. A comparison was made between subunits dissociated by chelating agents (EDTA) or by high concentrations of monovalent cations (0.85M KCl), and subunits isolated by either of the above methods were assayed for contamination by their complementary subparticle or by non-specific cytoplasmic proteins. Similarly, an assessment was made of the degree of cross-contamination of nucleolar particles after their isolation by the method of Warner and Soeiro (1967). Furthermore, prior to investigation of the mechanism of ribosome assembly in the nucleolus, the ratio of protein/RNA found in nucleolar particles and mature ribosomal subunits was determined by CsCl buoyant density centrifugation studies. Finally, polyacrylamide gel electrophoresis was used to find the distribution of methionine through different ribosomal subunit proteins. This was an important preliminary experiment as it indicated whether the radioactive marker (L-[^{35}S]methionine) used in subsequent two dimensional finger-printing studies of ribosomal proteins would appear, after labelling, in all or most proteins, or whether it would be confined to one or a few, possibly unrepresentative, polypeptides.

(1) Comparison of methods of ribosome dissociation: Dissociation by EDTA and by 0.85M KCl.

Eukaryotic ribosomes may be dissociated into subunits either by elimination of magnesium by the use of chelating agents (e.g. EDTA) (Hamilton and Petermann, 1959; Tashiro and Siekevitz, 1965), or by addition of high concentrations of competing monovalent cations (Martin and Wool, 1968).

Fig. III (1) shows the result of an experiment to compare HeLa cell ribosome dissociation by EDTA and by 0.85M KCl treatment. The advantages of EDTA

Fig. III (1) Comparison of methods of ribosome preparation. EDTA vs. 0.85M KCl as dissociation agent

Two cultures of 25 x 10^6 HeLa monolayer cells in 200ml EC 10 were incubated at 37°C for 36 hours after addition of 1μCi [^{14}C] uridine (60mCi/mmol) and 250μCi L-[^{3}H]leucine (final specific activity = 12.5mCi/mmol). The cells were harvested by scraping and ribosomes prepared by magnesium precipitation as described in METHODS. The ribosomes were split into two equal aliquots, A and B. A ribosomes were suspended in 1.0ml of NEB and separated into subunits in 36ml 15-30% sucrose gradients in NEB.

B ribosomes were suspended in 1.0ml of 0.85M KCl buffer and also separated into subunits in 36ml 15-30% sucrose gradients in 0.85M KCl buffer. Centrifugation was performed in a Beckman L2 ultracentrifuge with an SW 27 rotor (17 hours, 80,000g, +4°C). The gradients were collected into approximately 30 fractions which were made 10% with TCA. Acid insoluble material was collected on membrane filters and radioactivity determined in a Packard Tricarb liquid scintillation spectrometer using toluene based scintillant.

Technical details are provided in METHODS.

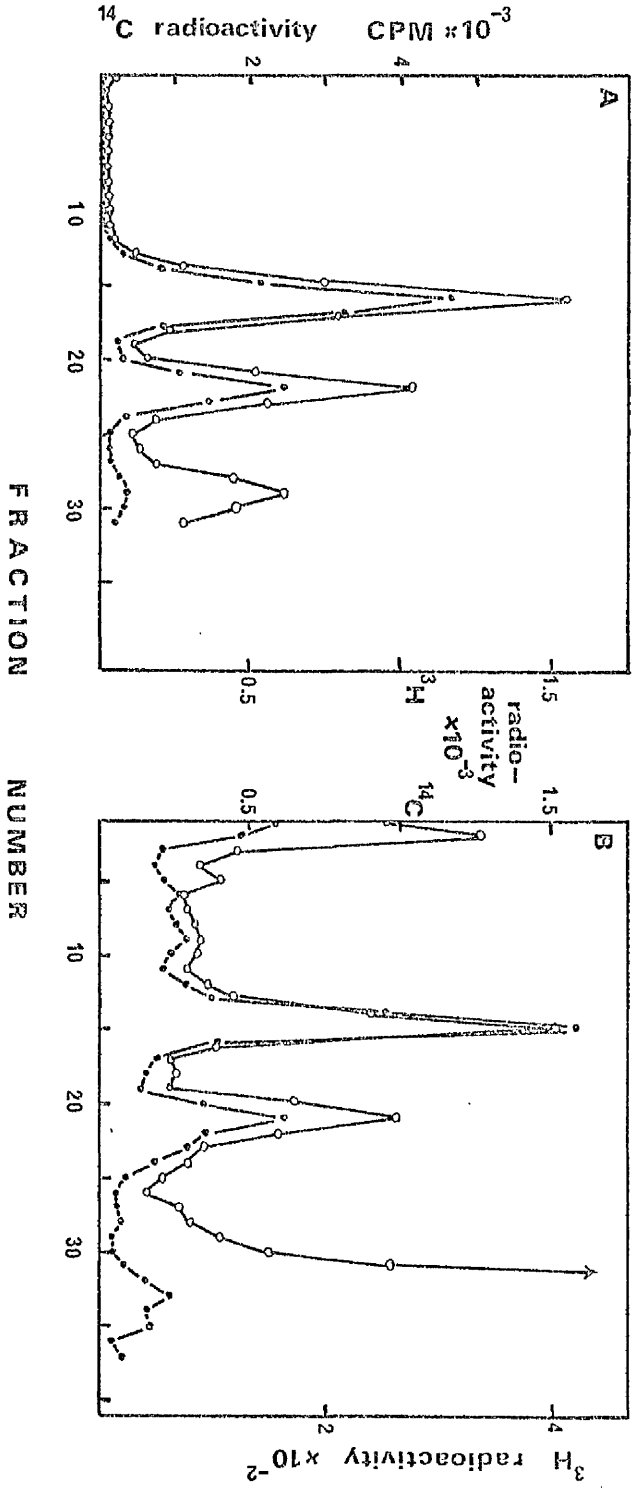
Panel A: EDTA dissociated ribosomal subunits

Panel B: 0.85M KCl dissociated ribosomal subunits

Sedimentation is from right to left.

Acid insoluble [^{3}H] radioactivity —o—o—

Acid insoluble [^{14}C] radioactivity —⊙—⊙—


^{14}C radioactivity CPM $\times 10^{-3}$
A
B
^{3}H
^{14}C
radio-activity $\times 10^{-3}$
^{3}H radioactivity $\times 10^{-2}$
FRACTION NUMBER

dissociation appear to be threefold. First, dissociation is complete (Fig. III (1)A), and the resulting subunits show minimal cross-contamination on recentrifugation (see Expt. III A (2)). Secondly, the method gives consistently good yields of subunits. Thirdly, later experiments in this study employ EDTA to prepare nucleolar particles. Therefore, for comparative purposes, ribosomal subunits should also be prepared by EDTA dissociation. However, EDTA-dissociated subunits are not active in protein synthesis (Tashiro and Morimoto, 1966), and therefore it is uncertain whether they contain all proteins necessary for ribosome activity. Moreover, they may be contaminated with adsorbed non-ribosomal proteins (Warner, 1966).

Conversely, treatment of ribosomes with high concentrations of various salts results in protein detachment (Itoh et al., 1968; Spitnik-Elson and Atsmon, 1969; Clegg and Arnstein, 1970; Reboud et al., 1971). The ribosomes, however, after KCl treatment, remain active in protein synthesis (Martin and Wool, 1968) and therefore may be assumed to contain at least the minimum number of proteins consistent with ribosome activity. The main disadvantage of KCl dissociation is that ribosomes from some species are resistant to dissociation by this means (Martin and Hartwell, 1970) and, in our hands, ribosomal subunit separation by KCl was incomplete and resulted in cross-contamination of large with small subunit material (see Fig. III (1) B; Fig. III (3) and Fig. III (11)). Such cross-contamination may have arisen by dimerisation of small ribosomal subunits which then cosediment with their larger counterpart (Hamilton et al., 1971). Alternatively, the contamination may reflect the relative resistance of active (i.e. messenger-associated) ribosomes to 0.85M KCl dissociation (Zylber and Penman, 1970). Such a phenomenon would explain the low yield of subunits obtained by KCl dissociation, and contamination of large with small subunit material would result from the presence of undissociated mono- or polyribosomal material in the preparative sucrose gradients (the persistence of material of sedimentation coefficient greater than 60s in

	Ratio of protein radioactivity in large/small subunit (4 experiments)	Ratio of RNA radioactivity in large/small subunit (3 experiments)
EDTA dissociation	1.69	2.40
0.85M KCl dissociation	1.16	2.35

Table III (1) Comparison of methods of ribosome preparation. EDTA vs. 0.85M KCl as dissociation agent.

Experiments were performed as in the legend of Fig. III (1) and ratios of large/small subunit radioactivity determined.

Fig.III (1) B substantiates this postulate). Moreover, ribosome dissociation by high concentrations of monovalent cations is dependent partly on the gravitational force applied to the particle. Reduction of this force results in incomplete dissociation, particle dimerisation and probably subunit cross-contamination (Infante and Krauss, 1971; Infante and Baierlein, 1971).

Table III (1) summarises a number of comparative studies of EDTA vs 0.85M KCl ribosome dissociation. Note that the ratio of radioactive RNA in large/small subunits is independent of the method of dissociation, whereas the ratio of protein radioactivity in large/small subunits is higher for EDTA- than for KCl-dissociated ribosomal particles. This result, coupled with that of Expt. III A (5) indicates that there is specific loss of large subunit protein during 0.85M KCl dissociation (see also Ford, 1971). If it is assumed that small ribosomal subunits dissociated by either method have the same RNA/protein ratio (confirmed by Expt. III A (5)), it can be calculated that the 0.85 M KCl-dissociated large subunit contains approximately 30% less protein than its EDTA-derived counterpart, and has a complement of 66% RNA (EDTA dissociated subunits contain 49-51% RNA - Expt. III A (5)). This result is in good agreement with the calculated percentage RNA derived by CsCl buoyant density centrifugation of the 60s subunit (see Expt. III A (5)).

The large amount of supernatant protein found in the 0.85M KCl - sucrose gradient relative to that in the EDTA gradient (Fig. III (1)) is consistent with the conclusion that proteins are stripped from the 60s HeLa ribosomal particle during or prior to centrifugation through KCl.

(2) Efficiency of ribosomal subunit separation by sucrose gradient sedimentation.

It is essential in chemical and physical investigations of protein structure that pure proteins are studied. The nature of the two dimensional peptide fractionation procedure used in this study, however, made absolute purification of ribosomal subunit proteins unnecessary. Moreover, contamination of one subunit protein complex with trace amounts of the

corresponding subunit proteins would not be detected by the fingerprinting method.

Figs. III (2) and (3) show the results of experiments in which ribosomal subunits were recentrifuged after separation in EDTA- or 0.85M- containing sucrose gradients. EDTA-dissociated subunits show minimal cross-contamination (Fig. III (2); approximately 4.0-4.5% contamination of one subunit with the other), which was considered to be acceptable for the purpose of this study. Conversely, 60s KCl-dissociated particles were found to be 7.3% contaminated with 40s material (Fig. III (3)), a result which appears to impose restrictions on the interpretation of later two dimensional fingerprinting studies of 60s subunit proteins (see Fig. III (11)). Contamination of 40s by 60s subunits was negligible (2.6%).

(3) Determination of the degree of ribosomal subunit contamination with non-specific cytoplasmic proteins.

It is important in studies of ribosomal structural proteins to exclude the possibility that a fortuitous association may occur between non-ribosomal cytoplasmic proteins and ribosomal subunits during their isolation from the cell. This question was investigated by resuspending magnesium-precipitated ribosomes in [^{14}C] leucine-labelled cytoplasm from which the ribosomes had been removed by high speed centrifugation. Ribosome reprecipitation and separation into subunits on sucrose gradients containing EDTA or 0.85M KCl as dissociating agent indicated (Fig. III (4)) that negligible extraneous protein contamination of ribosomes occurred during the isolation procedure.

(4) Nucleolar particle purification

Warner and Soeiro (1967) and Liau and Perry (1969) have shown that ribonucleoprotein particles extracted from HeLa and L cell nucleoli contain the precursors of ribosomal RNA. Using a modification of the technique described by Warner and Soeiro (see METHODS), these particles were derived from HeLa cells and separated into two classes (80s and 55s) on sucrose gradients (Fig. III (5) A). Pooled peak fractions were concentrated by

Fig. III (2) Purity of EDTA dissociated subunits isolated on sucrose gradients

A culture of 25 x 10^6 HeLa monolayer cells in 200ml EC 10 was incubated for 19 hours at 37°C after addition of 25µCi L-[^{14}C] leucine (final specific activity = 1.25mCi/mmol). The cells were harvested by trypsinisation, ribosomes isolated, dissociated by EDTA treatment, and separated into subunits in a 36ml 15-30% sucrose gradient containing NEB (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The gradient 50s and 30s peak fractions were pooled (Fig. III (2) A), and dialysed separately for 16 hours at 0°C against a 250 fold excess of 30% polyethylene glycol (mol. wt. = 20,000) in NEB. The dialysed and concentrated subunit fractions were layered over 16ml 15-30% sucrose gradients in NEB and recentrifuged as before. Unlabelled "marker" HeLa ribosomes were sedimented concurrently in a separate gradient. The gradients were collected into approximately 30 fractions, each fraction made 10% with TCA, acid insoluble material collected on membrane filters, and radioactivity determined in a Nuclear Chicago low background gas flow counter.

Details of fractionation and centrifugation procedures are given in METHODS.

Panel A: Absorbance profile of ribosomes showing the pooled peak fractions taken

Panel B: Radioactivity profile of recentrifuged 50s material.

Panel C: " " " " 30s "

The arrows in B and C indicate the position of 50s "marker" ribosomal subunit material sedimented concurrently in a separate gradient.

Sedimentation is from right to left.

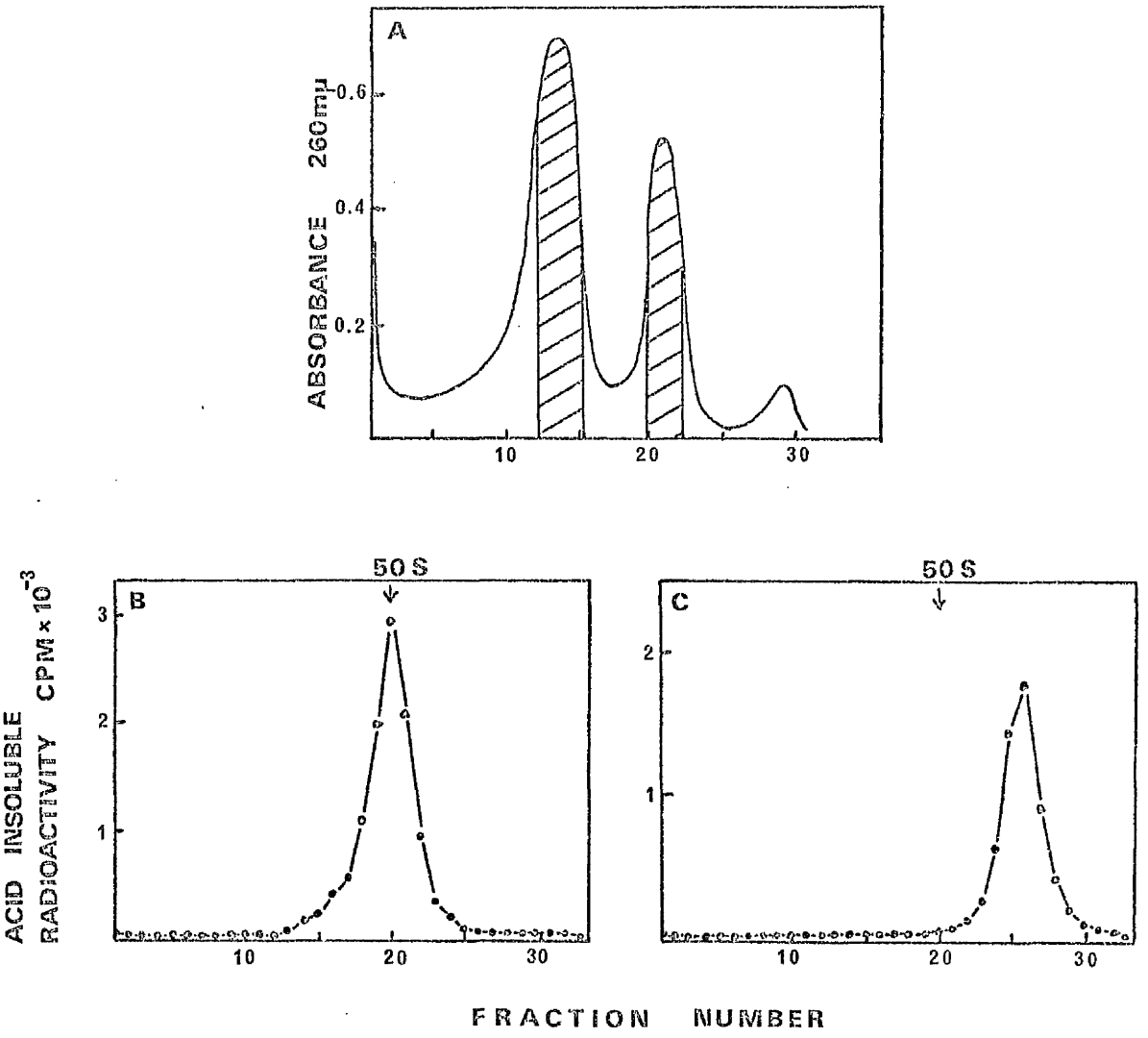

A
ABSORBANCE 260mμ
0.6
0.4
0.2
10
20
30
50 S
B
C
ACID INSOLUBLE RADIOACTIVITY CPM × 10⁻³
3
2
1
FRACTION NUMBER

Fig. III (3) Purity of 0.85M KCl dissociated ribosomal subunits isolated on sucrose gradients

Approximately 10^8 HeLa monolayer cells were used to prepare ribosomes by magnesium precipitation. The ribosomes were dissociated using 0.85M KCl buffer, and subunits separated in 36ml 15-30% sucrose gradients containing 0.85M KCl buffer (17 hours, 80,000g, +4°C using a Beckman L2 ultracentrifuge and SW 27 rotor). 60s and 40s peak fractions were pooled and the ribosomal subunits precipitated by addition of polyethylene glycol (mol. wt. 6,000) to 10%. After standing for 2 hours at 0°C, the precipitated ribosomes were collected by centrifugation (30 minutes, 15,000g, 0°C) and resuspended in 1.0ml of NEB. The subunits were resedimented through 20ml 15-30% sucrose gradients in NEB using an MSE High Speed 50 centrifuge and SW 30 rotor(16 hours, 80,000g, +4°C). After centrifugation, the optical density through each gradient was determined at 260mμ using a Gilford 240 spectrophotometer.

Technical details are provided in METHODS.

Panel A: Absorbance profile of 0.85M KCl-dissociated ribosomes showing peak fractions taken.

Panel B: Absorbance profile of 60s resedimented subunits

Panel C: " " " 40s " "

Sedimentation is from right to left.

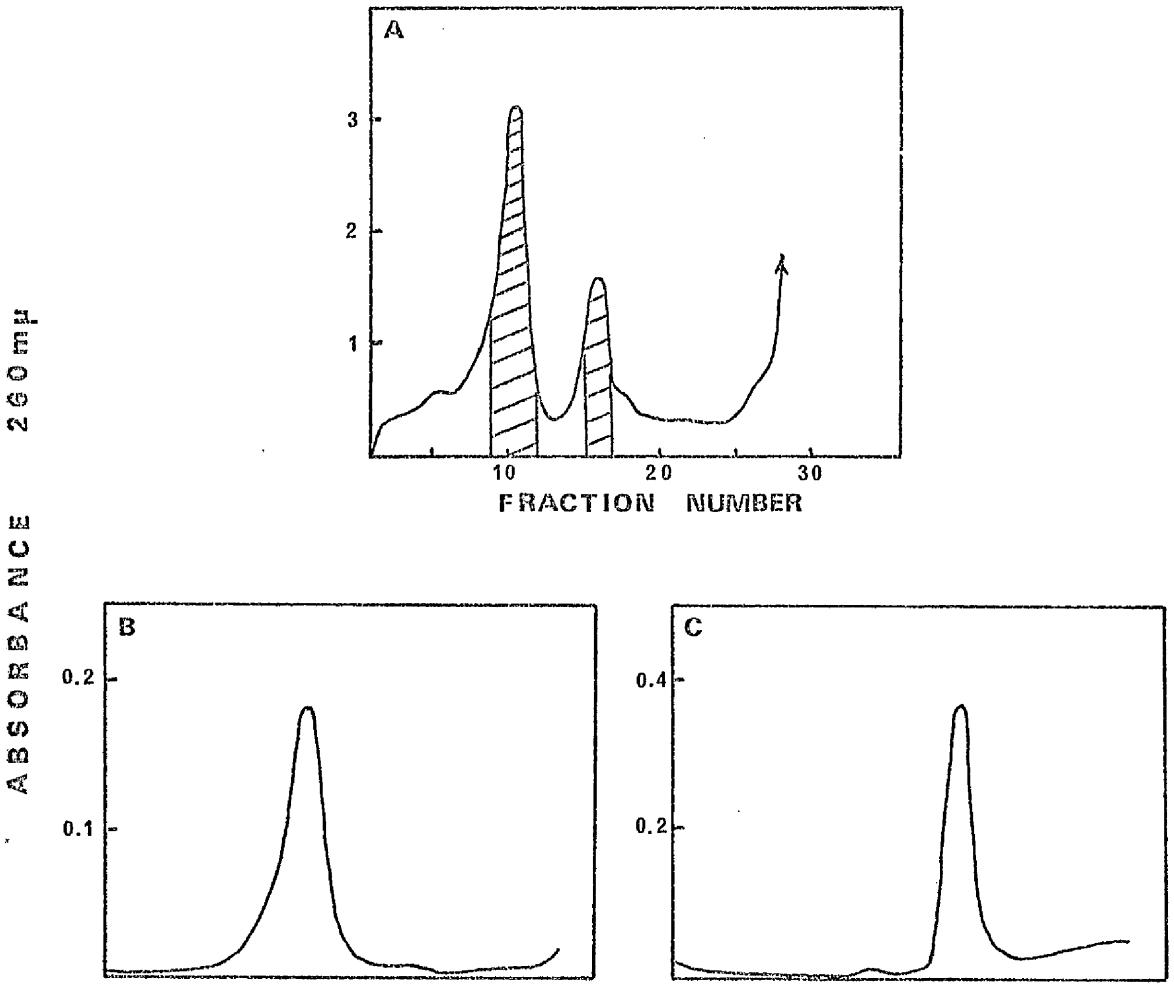

A
3
2
1
10
20
30
FRACTION NUMBER
ABSORBANCE 260mμ
B
0.2
0.1
C
0.4
0.2

Fig. III (4) Ribosomal subunit purity. Investigation of possible protein contamination during subunit isolation.

2 cultures of 25 x 10^6 HeLa monolayer cells were grown at 37°C in 200ml EC 10. To one was added 30μCi L-[^{14}C]leucine (final specific activity = 1.25mCi/mmol), and incubation was continued for 48 hours. Each culture was then harvested separately, and ribosomes were prepared by magnesium precipitation. The unlabelled and [^{14}C]leucine labelled cytoplasmic supernatants remaining after ribosome precipitation were cleared of residual ribosomes by high speed centrifugation in a Beckman L2 ultracentrifuge using a Ti 50 rotor (150,000g, 0°C, 120 minutes). Unlabelled ribosomes were resuspended in radioactive cytoplasmic supernatant and vice versa, and the ribosomes reprecipitated with magnesium as in METHODS. The reprecipitated ribosomes were split into two equal aliquots, A and B.

Aliquots A were suspended in 1.0ml NEB and the subunits separated in 36ml 15-30% sucrose gradients in NEB.

Aliquots B were treated likewise, except that 0.85M KCl buffer was used for resuspension and recentrifugation (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The gradients were harvested into approximately 30 fractions which were made 10% with TCA. Acid insoluble material was collected on membrane filters and counted in a Nuclear Chicago low background gas flow counter.

Experimental details are found in METHODS

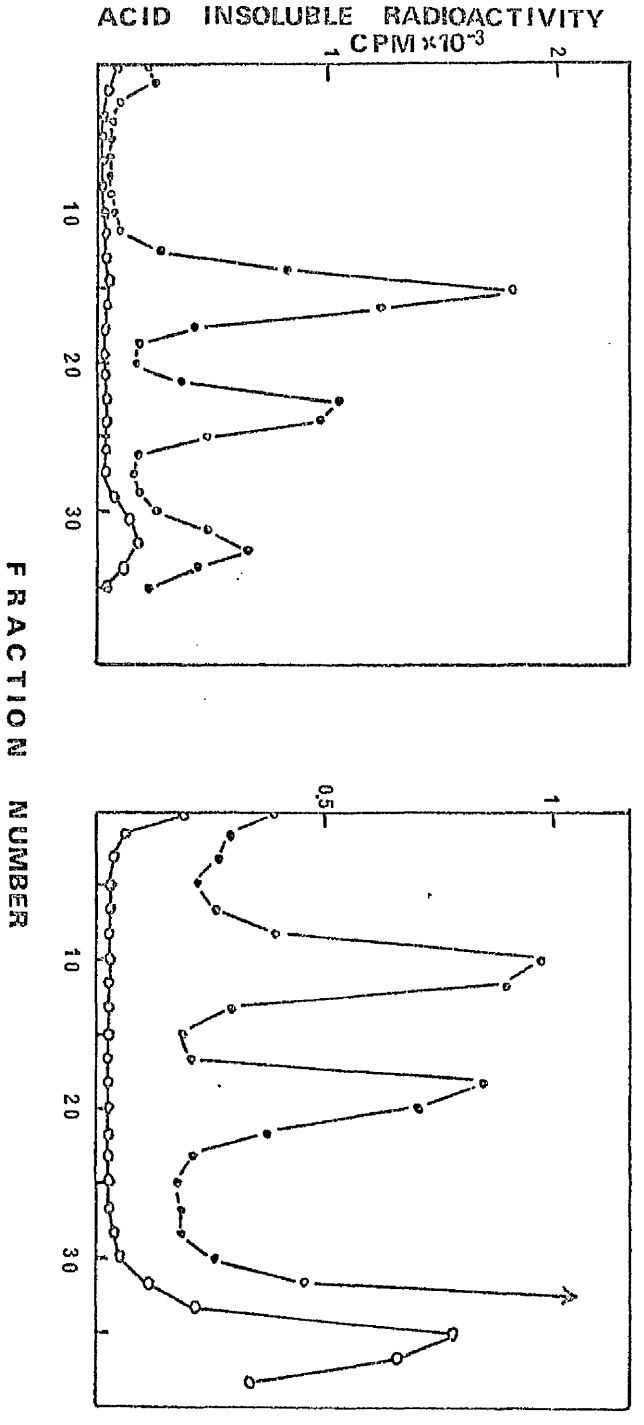
Panel A: EDTA dissociated ribosomes

Panel B: 0.85M KCl-dissociated ribosomes

[^{14}C] ribosomes reprecipitated from unlabelled cytoplasm —— ● —— ● ——

Unlabelled ribosomes precipitated from [^{14}C] cytoplasm —— ○ —— ○ ——

Sedimentation is from right to left.


ACID INSOLUBLE RADIOACTIVITY
CPM×10⁻³
2
1
0.5
10
20
30
FRACTION NUMBER

dialysis against 30% polyethylene glycol (mol.wt. = 20,000) and the purity of the particles in each peak determined by recentrifugation on sucrose gradients. The 80s nucleolar particle is evidently unstable, and, on recentrifugation, is degraded to two more slowly sedimenting moieties with "S" values of approximately 56 and 27 (Fig. III (5)B). The 55s particle shows less lability on recentrifugation, although the peak at 55s is skewed towards the top of the gradient, indicating the occurrence of some degradation (Fig. III (5)C).

Fixation of the particles with glutaraldehyde, which links covalently the primary amino groups of proteins and RNA bases, stabilises the 80s and 55s particles during recentrifugation (Figs. III (5)B and C). If it is assumed that stabilisation by glutaraldehyde is complete, and no degradation of the fixed particles occurs on recentrifugation, it can be estimated that contamination of the isolated 80s particles with 55s material is of the order of 27%. This result carries important implications for the interpretation of data from later experiments. Glutaraldehyde-fixed 55s particles are not degraded on recentrifugation and show negligible contamination with 80s material.

(5) Buoyant densities of ribosomal subunits and nucleolar particles in caesium chloride.

The buoyant density of ribonucleoprotein particles is a reflection of their RNA/protein ratio (Perry and Kelley, 1966a; Hamilton and Ruth, 1969). It was desirable as a preliminary to detailed analysis of the proteins of ribosomal subunits and their precursors to determine the approximate protein content of these particles. This was done by analysing the particles using equilibrium centrifugation in CsCl gradients.

(a) Buoyant density of ribosomal subunits.

The legend of Fig. III (6) indicates the method used to determine the buoyant densities of large and small ribosomal subunits prepared by EDTA- and 0.85M KCl-dissociation. Table III (2) shows comparative buoyant densities

Fig. III (5) Stabilisation of nucleolar particles by glutaraldehyde fixation

1.6×10^8 HeLa cells were labelled for 30 minutes at 37°C in 60ml leucine-deficient Joklik modified EC 10 with 750μCi L-[^{3}H]leucine (final specific activity = 19Ci/mmol). The cells were harvested and used to prepare nucleolar particles as described in METHODS. The particles were separated on a 36ml 15-30% sucrose gradient in NEB (16 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The peak fractions were pooled (Fig. III (5) A) and split into equal aliquots A and B. Aliquots A were treated with 8% glutaraldehyde according to the procedure of Baltimore and Huang (1968) - see METHODS. Aliquots B were untreated. The four fractions (80sA, 80sB, 55sA, 55sB) were then dialysed separately against a 250 fold excess of 30% polyethylene glycol (mol. wt. = 20,000) in NEB for 6 hours at 0°C , and after addition of unlabelled "marker" HeLa ribosomes, the aliquots were resedimented in 16ml 15-30% sucrose gradients in NEB (16 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The gradients were harvested into approximately 30 fractions which were made 10% with TCA. Acid insoluble material was collected on membrane filters, toluene based scintillant added, and radioactivity determined in a Packard Tricarb liquid scintillation spectrometer.

Panel A: Absorbance profile of nucleolar particles, showing peak fractions taken.

Panel B: Resedimented unfixed —o—o— and glutaraldehyde fixed —O—O— 80s material.

Panel C: Resedimented unfixed —o—o— and glutaraldehyde fixed —O—O— 55s material.

The arrows indicate the position of "marker" 50s ribosomal subunits.

Sedimentation is from right to left.

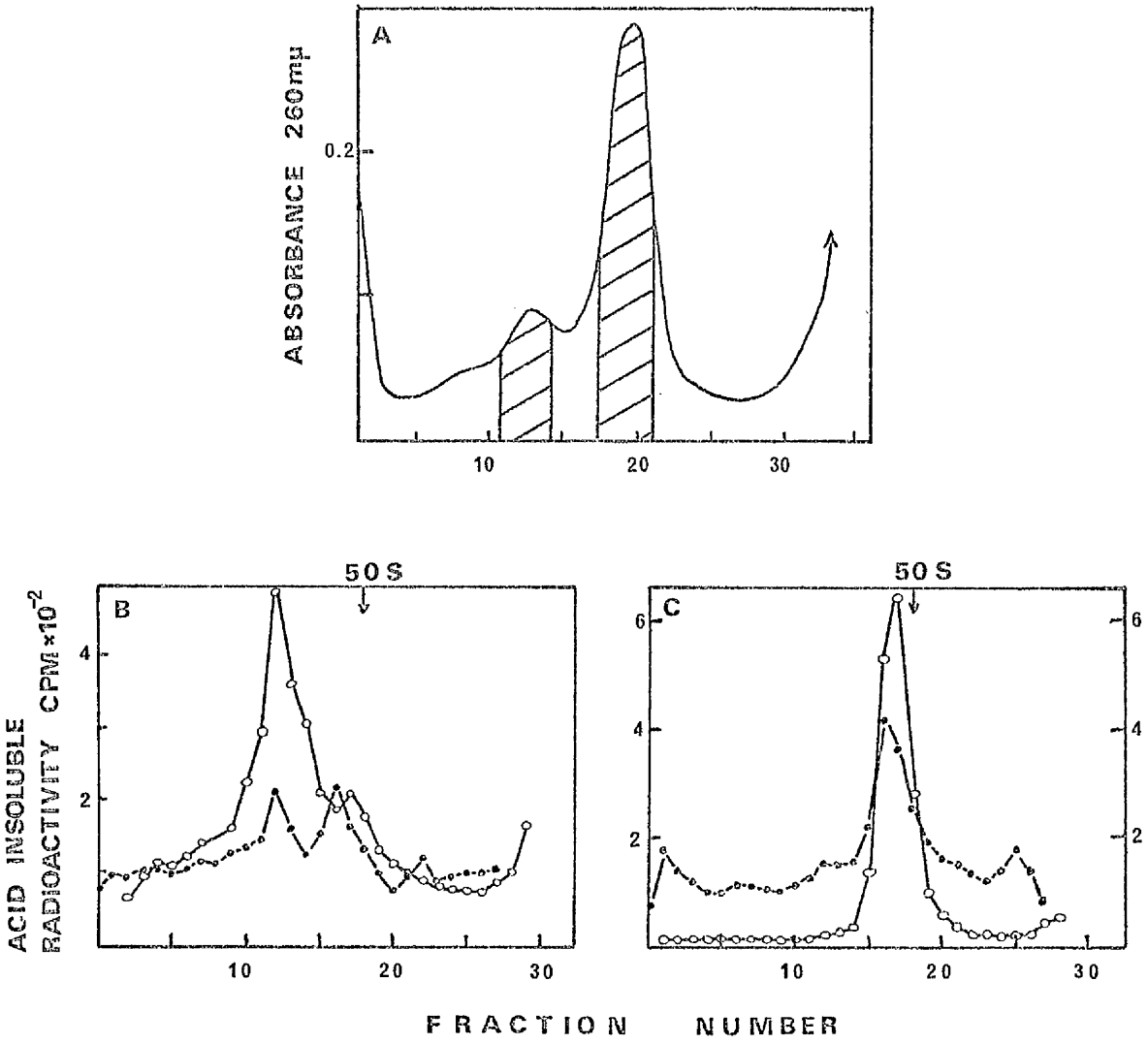

A
ABSORBANCE 260mμ
0.2
10
20
30
50S
B
C
ACID INSOLUBLE RADIOACTIVITY CPM×10⁻²
4
2
6
FRACTION NUMBER

Fig III (6) Buoyant densities of EDTA- and 0.85M KCl-derived HeLa cell ribosomal subunits

(1) Buoyant density of EDTA-derived ribosomal subunits

Ribosomes were prepared from a 25 x 10^6 cell HeLa monolayer culture grown in 200ml of EC 10 for 48 hours at 37°C in the presence of 200μCi L-[^{3}H]leucine (final specific activity = 10mCi/mmol). The ribosome pellet was dissociated into subunits by suspending in 1.0ml of NEB, and the subunits separated on a 16ml 15-30% sucrose gradient in NEB (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Peak fractions were pooled (Fig. III (6) A), glutaraldehyde added to 8% (Baltimore and Huang, 1968) and subunits pelleted by centrifugation (5 hours, 150,000g, 0°C in a Beckman L2 ultracentrifuge with Ti 50 rotor). The pelleted subunits were suspended, with gentle homogenisation in 0.2ml of RSB, layered over 5ml preformed 33-55% (w/w) CsCl gradients in RSB, and centrifuged to equilibrium in an MSE High Speed 50 centrifuge with an SW 40 rotor(16 hours, 100,000g, +4°C) The gradients were collected into approximately 30 split fractions by piercing the bottom of the tube as described in METHODS Section (5)a. The refractive index of the gradient fractions was measured on 1/5th of each fraction using an Abbe refractometer and related to densities (Vinograd and Hearst, 1962). The remainder of each fraction was made 10% with respect to TCA, acid insoluble material collected on membrane filters, toluene based scintillant added and radioactivity determined in a Packard Tricarb spectrometer.

Panel A: Absorbance profile of EDTA dissociated subunits, showing peak fractions taken.

Panel B: Radioactivity profile of 50s subunits centrifuged to equilibrium in CsCl.

Panel C: Radioactivity profile of 30s subunits centrifuged to equilibrium in CsCl.

(2) Buoyant density of 0.85M KCl-derived ribosomal subunits

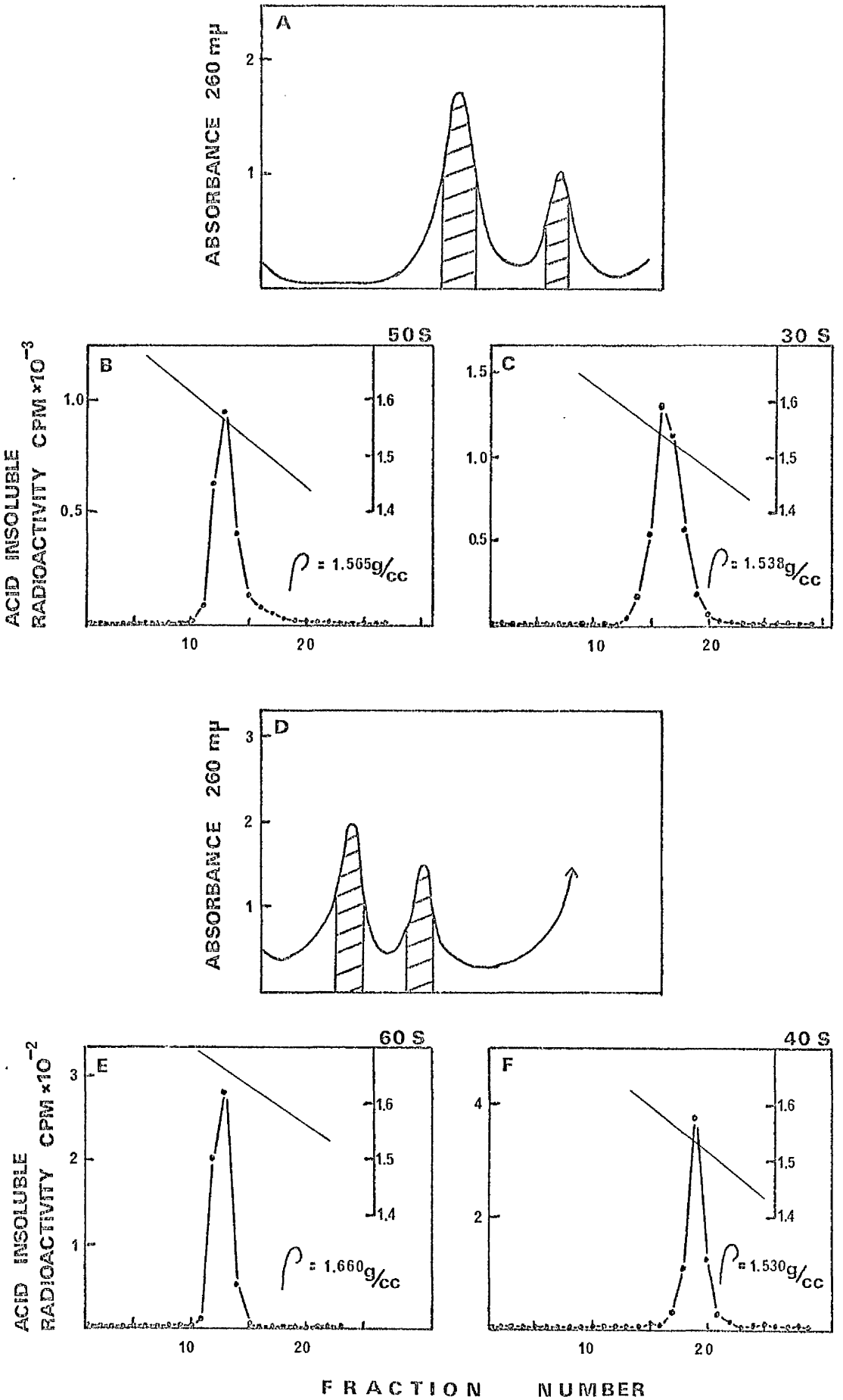
Ribosomes were prepared from a 25 x 10^6 HeLa monolayer culture grown in 200ml EC 10 for 48 hours at 37°C in the presence of 500μCi L-[^{3}H]phenylalanine (final specific activity = 7mCi/mmol). Subunits were dissociated by suspension in 1.0ml of 0.85M KCl buffer and separated in sucrose gradients containing 0.85M KCl buffer using the experimental conditions described above for EDTA dissociated subunits. Pooled peak fractions (Fig. III (6) D), after fixation in 8% glutaraldehyde, were dialysed and concentrated simultaneously against a 250 fold excess of 30% polyethylene glycol (mol. wt. 20,000) in RSB. The buoyant densities of the concentrated 40s and 60s subunit material were determined by centrifugation to equilibrium in CsCl as described above for EDTA dissociated subunits.

Panel D: Absorbance profile of 0.85M KCl-dissociated subunits showing pooled peak fractions

Panel E: Radioactivity profile of 60s subunits centrifuged to equilibrium in CsCl.

Panel F: Radioactivity profile of 40s subunits centrifuged to equilibrium in CsCl.

In all cases, sedimentation is from right to left.


A
ABSORBANCE 260 mμ
2
1
50 S
B
ACID INSOLUBLE RADIOACTIVITY CPM ×10⁻³
1.0
0.5
1.6
1.5
1.4
ρ = 1.565g/cc
10
20
30 S
C
1.5
1.0
0.5
1.6
1.5
1.4
ρ = 1.538g/cc
10
20
D
ABSORBANCE 260 mμ
3
2
1
60 S
E
ACID INSOLUBLE RADIOACTIVITY CPM ×10⁻²
3
2
1
1.6
1.5
1.4
ρ = 1.660g/cc
10
20
40 S
F
4
2
1.6
1.5
1.4
ρ = 1.530g/cc
10
20
FRACTION NUMBER

	Buoyant Densities (g/cc)	
	Large Subunit	Small Subunit
EDTA dissociation	1.565	1.538
0.85M KCl dissociation	1.660	1.530
	% protein per subunit	
	Large Subunit	Small Subunit
EDTA dissociation	52.2 (49)	57.5 (58)
0.85M KCl dissociation	34.3 (35)	59.4 (58)

Table III (2) Buoyant densities of ribosomal subunits prepared by EDTA and 0.85M KCl dissociation.

Subunit buoyant densities were determined as described in the legend of Fig. III (6). Percentage protein in the subunits was calculated by the method of Ford (1971). Figures in parenthesis are Ford's (1971) results for Xenopus ovary ribosomes.

of EDTA- and KCl-derived large ribosomal subunits, with calculated protein percentages per subunit.

The buoyant densities of large ribosomal subunits are higher than corresponding small subunit densities. This has also been demonstrated by other workers (Perry and Kelley, 1966a); Infante and Nemer, 1968; Belitsina et al., 1968). Small subunits isolated by EDTA or KCl treatments have similar buoyant densities, as noted by Ford (1971) for Xenopus ovary ribosomes. However, the KCl-derived 60s subunit has a higher buoyant density than its EDTA-dissociated counterpart, indicating that there is protein loss from the 60s particle during its isolation. The results from Expts. III A (1) and III A (5) show good correlation and demonstrate that percentage protein in the 60s (KCl-dissociated) subunit is lower than in the 50s (EDTA-derived) particle by approximately 30%. The small subunit is stable both to KCl and EDTA treatment.

From these data it has been inferred that the large ribosomal subunit derived from HeLa cells by EDTA dissociation contains loosely-bound protein which is "stripped" by 0.85M KCl. The proteins of the small subunit are resistant to "stripping" by this treatment.

(b) Buoyant density of nucleolar particles.

The buoyant densities of 80s and 55s HeLa nucleolar particles were determined as described in the legend of Fig. III (7). Table III (3) shows the buoyant densities obtained and also provides an estimate of the percentage protein in the particles (after Ford, 1971).

A number of points arise from these results:-

(i) The buoyant density of the 55s particle was determined on essentially pure material, while the 80s material used was approximately 20% contaminated with 55s particles (Fig. III (7) D and E shows sucrose gradient profiles of the isolated nucleolar particles, recentrifuged after glutaraldehyde fixation).

(ii) 80s and 55s nucleolar particles show a wider spread of buoyant density

Fig. III (7) Buoyant densitites of HeLa cell nucleolar particles.

Nucleolar particles were prepared from 2.5 x 10^8 HeLa S3 cells labelled for 30 minutes in 100ml leucine deficient Joklik modified EC 10 containing 500μCi L-[^{3}H]leucine (final specific activity = 5Ci/mmol). The particles were separated on a 36ml 15-30% sucrose gradient in NEB (17 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Peak fractions were pooled (Fig. III (7)A), made 8% with glutaraldehyde, and dialysed for 6 hours at 0°C against a 250 fold excess of 30% polyethylene glycol (mol. wt. = 20,000) in RSB. The dialysed and concentrated 80s and 55s material was split into two fractions, A and B. Fractions A were layered over 5ml preformed 33-55% (w/w) CsCl gradients in RSB and centrifuged to equilibrium in the SW 40 rotor of an MSE High Speed 50 centrifuge (16 hours, 100,000g, +4°C). The gradients were collected into approximately 30 split fractions and the refractive index of 1/5th of each fraction measured on an Abbe refractometer and related to densities (Vinograd and Hearst, 1962). The remainder of each fraction was made 10% with TCA, acid insoluble material collected on membrane filters and radioactivity estimated in a Packard Tricarb spectrometer using toluene based scintillant.

To fractions B were added unlabelled "marker" HeLa ribosomes and the material separated in 36ml 15-30% sucrose gradients in NEB (17 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The gradients were collected into approximately 30 fractions, the fractions made 10% with TCA, and acid insoluble material collected on membrane filters. Radioactivity in each fraction was assayed in a Packard Tricarb spectrometer using toluene based scintillant.

All experimental details are found in METHODS.

Panel A: Absorbance profile of nucleolar particles showing peak fractions taken.

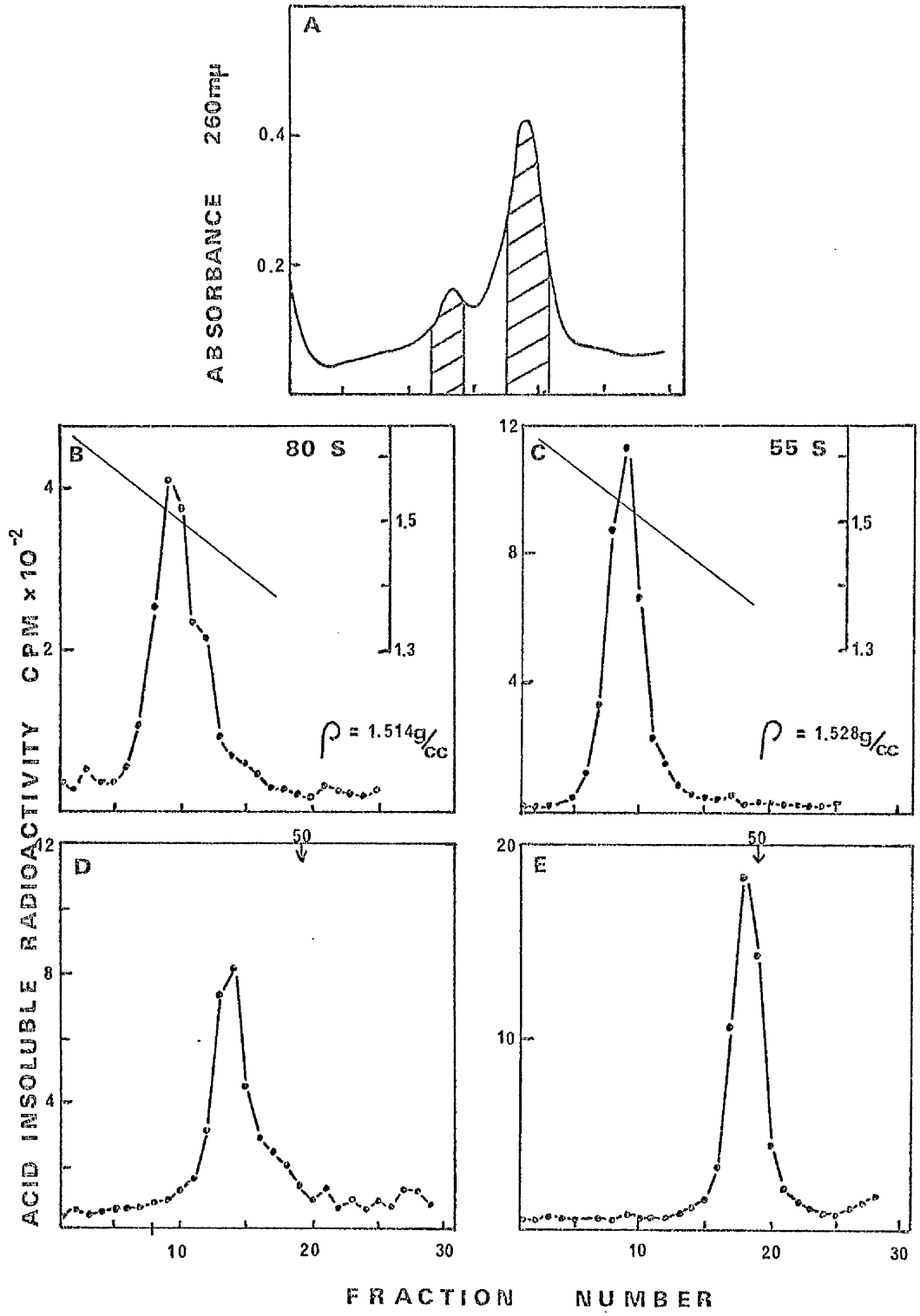
Panel B: Radioactivity profile of 80s material sedimented to equilibrium in a CsCl gradient.

Panel C: Radioactivity profile of 55s material sedimented to equilibrium in a CsCl gradient.

Panel D: Resedimentation of glutaraldehyde fixed 80s material purified on a sucrose gradient.

Panel E: Resedimentation of glutaraldehyde fixed 55s material purified on a sucrose gradient.

In all cases, sedimentation is from right to left.


A
ABSORBANCE 260mμ
0.4
0.2
B
80 S
1.5
1.3
ρ = 1.514g/cc
C
55 S
1.5
1.3
ρ = 1.528g/cc
D
E
50
50
ACID INSOLUBLE RADIOACTIVITY CPM x 10^-2
FRACTION NUMBER
10
20
30

	Buoyant Density (g/cc)	% Protein
80s particle	(L.1465 1.514((P.1520	62.8
55s particle	(L.1545 1.528((P.1550	59.4

Table III (3) Buoyant densities of HeLa cell 80s and 55s nucleolar particles.

The determinations were made as described in the legend of Fig. III (7). Percentage protein in the particles was calculated by the method of Ford (1971).

Nucleolar particle buoyant densities determined by Liau and Perry (1969) - "L" - and Pederson and Kumar (1971) - "P" - are shown.

than ribosomal subunits, suggesting the presence of a heterogeneous population of particles of varying RNA/protein content. This is particularly evident in the case of the 80s particle (Fig. III (7)B) and results, in part, from 55s contamination of the 80s material. Maturation or degradation of the 80s particle during its isolation may also be important in the production of the heterogeneous particle population (see Expt. III A (4)).

(iii) The buoyant density results indicate that there is progressive loss of protein from nucleolar particles as they mature to ribosomal subunits. This result has also been noted by other workers (Rogers, 1968; Liau and Perry, 1969; Craig and Perry, 1970; Pederson and Kumar, 1971).

(iv) Since approximately 50% of the 45s RNA is degraded during maturation to 28s and 18s rRNA (Weinberg and Penman, 1970), an even greater absolute protein loss during 80s particle maturation is implied than was at first evident from the buoyant density data.

(6) Distribution of methionine through HeLa ribosomal proteins.

Recent evidence suggests that methionine is found in most or all rat liver ribosomal proteins (Westermann et al., 1971). To confirm this observation, [^{35}S]methionine-labelled ribosomal proteins derived from HeLa cells were separated on SDS-acrylamide gels as described in METHODS. From the results (Fig. III (8)), it can be seen that most stained bands in the gel contain [^{35}S]methionine. Some proteins (gel bands 50s 1,7; 30s 1, 9, 15) are enriched for methionine, while a few (gel bands 50s 8, 10, 12; 30s 10, 11) show a relative deficiency of that amino acid. However, since [^{35}S]methionine appears in most gel bands, it has been concluded that a two dimensional [^{35}S]methionine-labelled fingerprint of ribosomal proteins would be altered by structural changes in almost any protein and would reflect the presence or absence of most protein components of the ribosome.

It should be noted that although the 50s and 30s gel patterns are

Fig. III (8) Distribution of L-[^{35}S]methionine through the proteins of HeLa cell ribosomal subunits

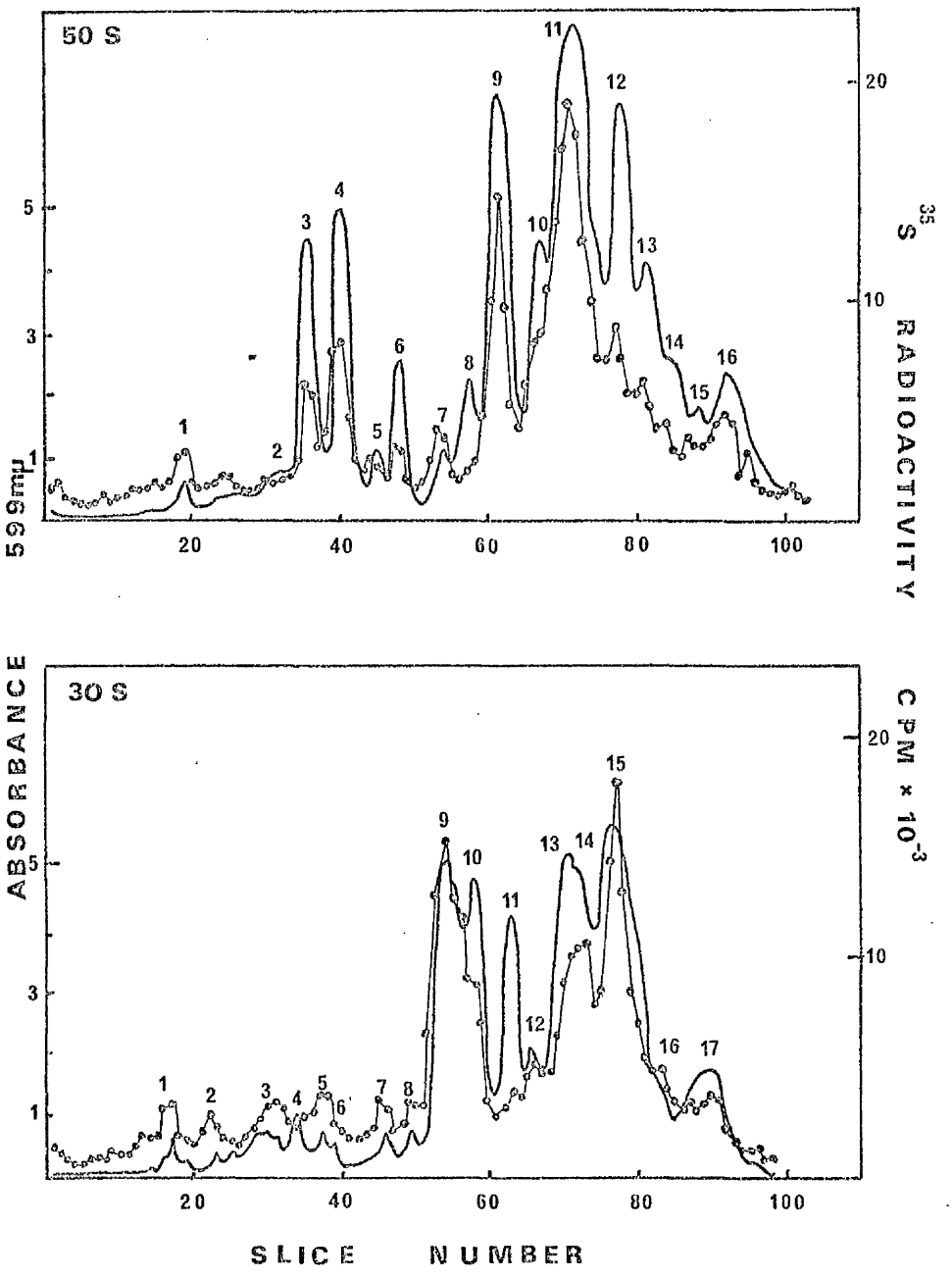
6×10^7 HeLa S3 cells were grown for 18 hours at 37°C in 200ml Joklik modified EC 10 medium in the presence of 500μCi L-[^{35}S] methionine (final specific activity = 25mCi/mmol). The cells were harvested, ribosomes prepared, dissociated into subunits using EDTA, and the subunits separated on 36ml 15-30% sucrose gradients in NEB (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The respective subunit peak fractions were pooled and the subunits ethanol precipitated. After dissolution of the ribosomal subunits in 0.2ml of gel sample buffer, the subunit proteins were examined by SDS-acrylamide gel electrophoresis according to the method of King et al. (1971). The gels were then stained, scanned at 599mμ in a Gilford 240 spectrophotometer, sliced into 1mm segments and the segments assayed for radioactivity.

All procedures are detailed in METHODS.

Absorbance at 599mμ ————————

[^{35}S] radioactivity —o— o—

The absorbance peaks obtained from 30s and 50s subunits have been numbered arbitrarily to facilitate description of the results.


50 S
599mμ
35S RADIOACTIVITY
30 S
ABSORBANCE
CPM × 10-3
SLICE NUMBER

recognisably different, incomplete resolution of bands on both gels, and coincident migration of many proteins from the large and small subunit render identification of proteins specific for each subunit extremely difficult, if not impossible. It was this limitation in ribosomal protein analysis by polyacrylamide gel electrophoresis which led to the development of the two dimensional protein fingerprinting method described in the next section.

Section III A Summary.

The results of the experiments described in this section may be summarised as follows :-

(1) HeLa cell ribosomes, dissociated by treatment with EDTA, are separable in sucrose gradients into subunits which show virtually no cross-contamination. Reconstruction experiments indicate that non-specific cytoplasmic proteins do not contaminate the particles during their isolation. Each subunit contains approximately 55% protein.

(2) Ribosome dissociation by 0.85M KCl is incomplete and results in approximately 7-8% contamination of large (60s) subunit with small (40s) subunit material. Moreover, although the small subunit has an RNA/protein ratio identical to that of its EDTA-derived (30s) counterpart, the large subunit loses approximately 30% of its protein during the 0.85M KCl-dissociation procedure.

(3) 80s particles, isolated by preparative sucrose gradient centrifugation, contain up to 27% contaminant 55s material as shown by recentrifugation studies. The 55s particles can, however, be isolated in relatively pure form from sucrose gradients.

(4) 80s and 55s particles are relatively labile on recentrifugation, but can be stabilised by fixation with glutaraldehyde.

(5) Nucleolar particles contain a higher percentage of protein than mature ribosomal subunits, and there appears to be progressive loss of protein from the particles during their maturation to cytoplasmic ribosomal subunits.

(6) Methionine is relatively widely distributed throughout the structural proteins of both ribosomal subunits, and few proteins appear to be either selectively enriched for, or completely deficient in this amino acid.

SECTION III B - Fingerprinting studies of the proteins derived from the HeLa cell ribosomes and their precursors.

Limited resolution of one dimensional polyacrylamide gel electrophoresis has made evident the need for a fractionation procedure to characterise the proteins of ribosomal subunits and their nucleolar precursors. This section describes the results obtained by the use of such a technique.

(1) Two dimensional fingerprinting studies of the HeLa cell ribosomal proteins labelled to equilibrium with [^{35}S]methionine.

(a) Fingerprints from EDTA-dissociated ribosomal subunits.

The two dimensional fingerprints of Fig. III (9) were obtained from [^{35}S] methionine-labelled proteins of ribosomal subunits prepared by EDTA dissociation and fractionated as described in METHODS. To permit identification of spots characteristic of either subunit, an equimolar mixture of large and small subunit proteins was also fingerprinted (Fig. III (9) C).

A number of points are evident from the fingerprints:-

(i) Both large and small subunit proteins produce a fairly large number of well resolved spots on fingerprinting.

(ii) the 30s and 50s patterns are markedly different from each other, few spots appearing to be common to both subunits.

(iii) There are considerable intensity differences between different spots on the same autoradiograph.

(iv) Repeat experiments, using constant digestion and fingerprinting conditions, indicate that the patterns are reproducible; and HeLa monolayer and S3 suspension cells also give fingerprints whose characteristic features are the same.

(v) Using the 30s/50s mixture fingerprint, several spots may be seen to be

Fig. III (9) Two dimensional peptide fingerprints of L-[^{35}S] methionine-labelled HeLa ribosomal subunits

4×10^7 HeLa monolayer cells were incubated for 72 hours at 37°C in 200ml EC 10 containing 2mCi L-[^{35}S]methionine (final specific activity = 80mCi/mmol). The cells were harvested by trypsinisation, ribosomes prepared, dissociated with EDTA and subunits separated on a 36ml 15-30% sucrose gradient in NEB (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The subunit peak fractions were pooled, the proteins extracted and fingerprinted according to the procedure in METHODS Section (4) C. The radioactive spots were located by autoradiography.

Technical details are described fully in METHODS.

Panel A: Two dimensional tryptic peptide fingerprint of HeLa 50s ribosomal subunit proteins.

Panel B: Two dimensional tryptic peptide fingerprint of HeLa 30s ribosomal subunit proteins.

Panel C: Two dimensional tryptic peptide fingerprint of an equimolar mixture of HeLa 30s and 50s ribosomal subunit proteins, used to detect subunit specific peptides.

Panel D: Two dimensional tryptic peptide fingerprint of cytoplasmic supernatant after removal of ribosomes by magnesium precipitation and high speed centrifugation (4 hours, 150,000g, +4°C in a Beckman L2 ultracentrifuge with Ti 50 rotor).

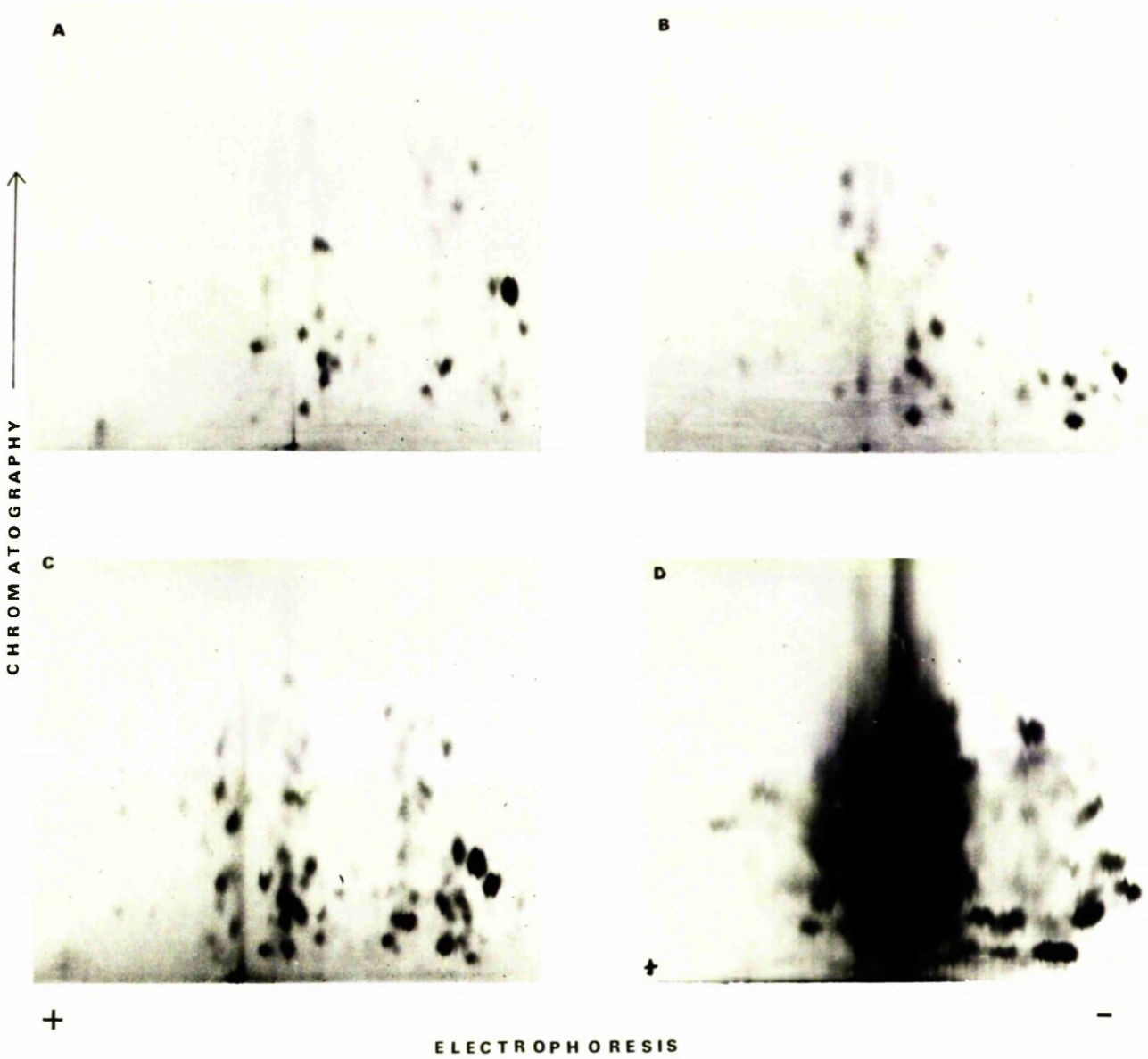

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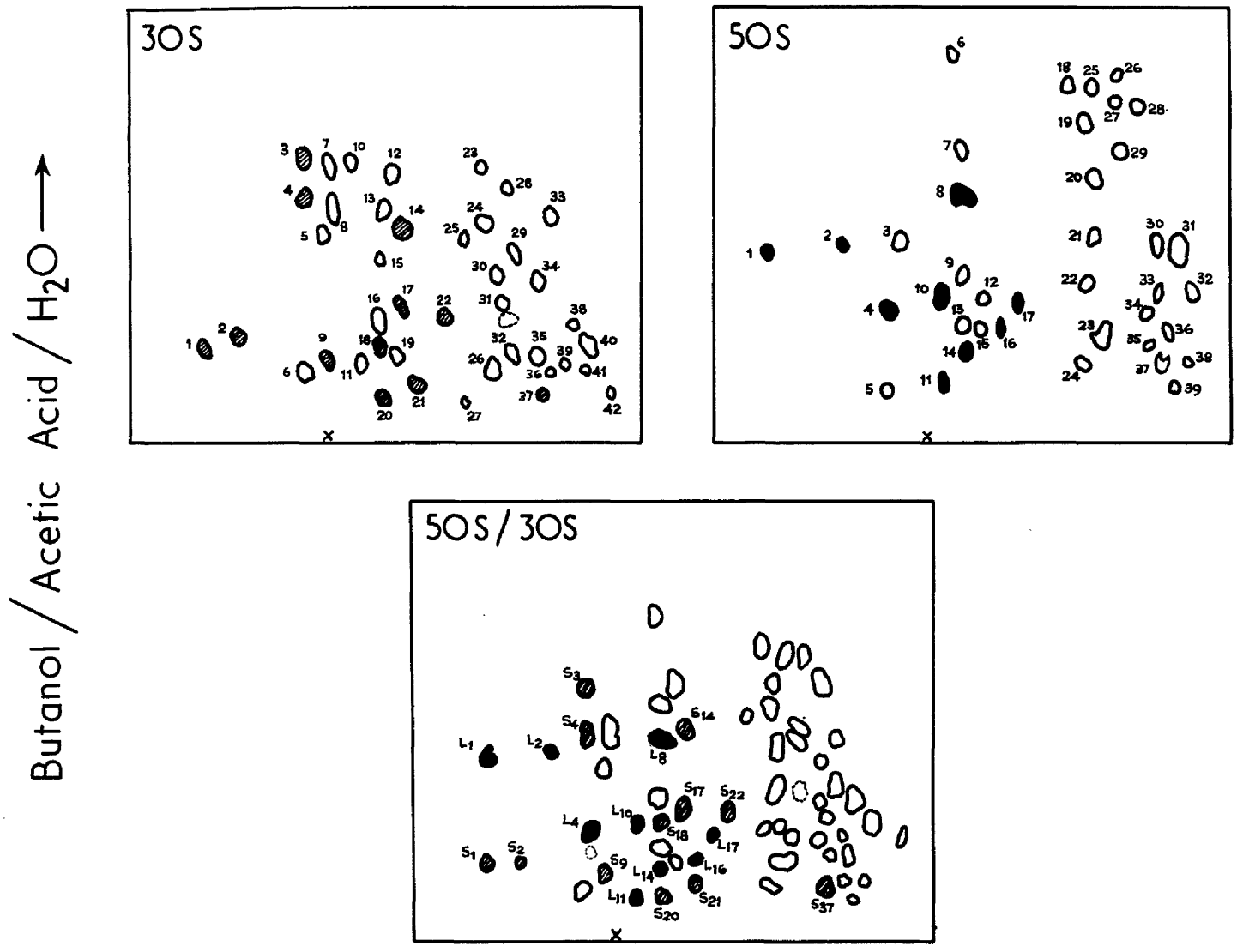
Fig. III (10). Reconstruction of the peptide fingerprints shown in Fig. III (9). Prominent and reproducible peptides in each subunit are numbered. The equimolar 50s/30s ribosomal protein mixture fingerprint was used to locate subunit-specific peptides.

Specific small subunit peptides 

Specific large subunit peptides 


30S
50S
50S / 30S
Butanol / Acetic Acid / H_2O
+ ELECTROPHORESIS pH 4·7 −

derived from one or other subunit. Fig. III (10) is a reconstruction of the fingerprints shown in Fig. III (9) with characteristic 50s and 30s spots numbered and identified as described in the legend. These spots were used as "landmarks" to indicate the presence or absence of large or small subunit peptides in ribosomal precursor particles. 30s/50s mixtures of molar ratio 1/3 contain, on fingerprinting, all 30s spots found on the 1/1 mixture, evidence that a threefold excess of 50s peptides does not mask the presence of 30s material.

(vi) The distinctive 30s and 50s patterns are in contrast to the complex pattern of incompletely resolved material obtained by tryptic digestion of "post ribosomal supernatant protein" (cytoplasm freed of ribosomes by high speed centrifugation - Fig. III (9) D).

(b) Fingerprints from 0.85M KCl-dissociated ribosomal subunits.

Salt-washing is a standard procedure used to free ribosomal subunits of contaminant protein (Spirin and Gavrilova, 1969). However, although salt-washed subunits are still active in protein synthesis (Martin and Wool, 1968), it has already been shown that large ribosomal subunits treated in this way lose 30% of their protein (Expts. III A (1) and (5). Fig. III (11) shows the result of a fingerprinting experiment in which ribosomes were dissociated by 0.85M KCl and separated on a 0.85M KCl-sucrose gradient to produce, effectively, a salt-wash. The ribosomes were prepared by magnesium precipitation as for Expt. III B (1)a, and so the results obtained in both experiments may be compared directly (cf. Figs. III (9) and (11)). The following points may be made:-

(i) Fingerprints of EDTA-dissociated and salt-washed ribosomes are similar, although the 60s subunit (KCl dissociated) pattern shows evidence of contamination with 40s peptides (60s fingerprint contains 40s peptides S9, 14, 27). This we ascribe to incomplete ribosome dissociation or 40s dimerisation when using the KCl-dissociation procedure (Martin and Hartwell, 1970; Zylber and Penman, 1970;

Fig. III (11) Comparison of peptide fingerprints obtained by EDTA- and KCl-dissociation of HeLa ribosomes

5×10^7 HeLa monolayer cells were grown for 70 hours in 200ml EC 10 containing 2mCi L-[^{35}S]methionine (final specific activity = 88mCi/mmol). The cells were harvested, ribosomes prepared by magnesium precipitation, dissociated in 0.85M KCl buffer and separated in a 36ml 15-30% sucrose gradient in 0.85M KCl buffer (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). 40s and 60s subunit peak fractions were pooled separately, the proteins extracted and fingerprinted according to the procedure described in METHODS Section (4) C. In this case, tryptic digestion was performed over 18 hours. The radioactive peptides on the fingerprints were located by autoradiography.

Technical details are described fully in METHODS.

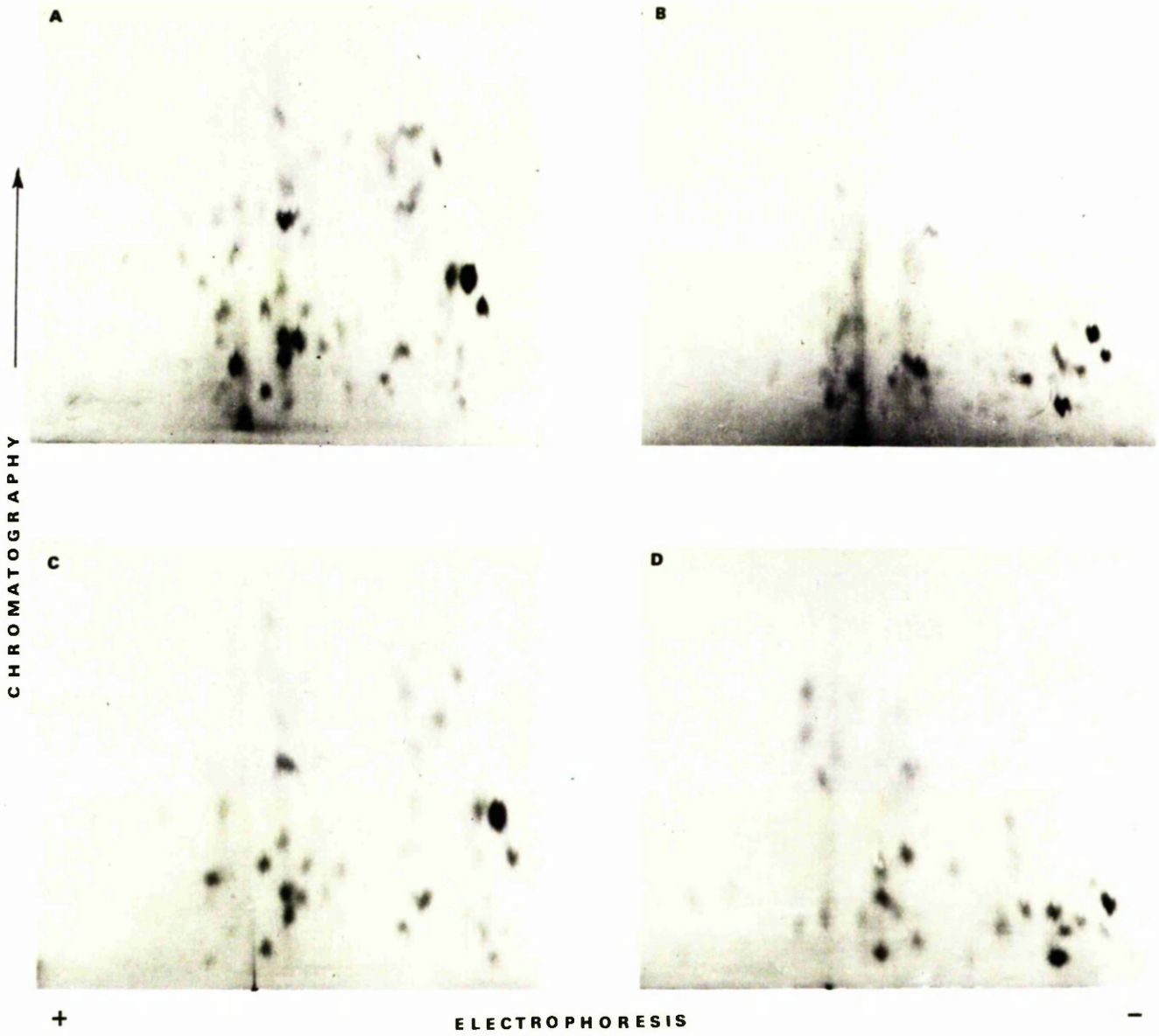
Panel A: Two dimensional tryptic peptide fingerprint of the proteins of 0.85M KCl-dissociated HeLa 60s ribosomal subunits.

Panel B: Two dimensional tryptic peptide fingerprint of the proteins of 0.85M KCl-dissociated HeLa 40s ribosomal subunits.

For comparative purposes EDTA dissociated HeLa cell ribosomal subunit fingerprints from Fig. III (9) have been included.

Panel C: Two dimensional peptide fingerprint of HeLa 50s ribosomal subunit proteins (EDTA dissociated).

Panel D: Two dimensional peptide fingerprint of HeLa 30s ribosomal subunit proteins (EDTA dissociated).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Infante and Baierlein, 1971; Infante and Krauss, 1971; Hamilton et al., 1971).

(ii) Excluding 40s contaminant spots on the 60s subunit fingerprint, some differences do exist between EDTA- and KCl-dissociated ribosomal subunit peptide patterns. These appear primarily to be the result of variations in spot intensity. For example, on the 60s subunit fingerprint (Fig. III (11) A), above spot L9 is a trio of spots, of which two are very faint and one absent on the 50s (EDTA-dissociated) "map". Another trio above spots L1 and L2 is very faint on the 50s subunit pattern. On the 40s subunit fingerprint (Fig. III (11) B), spots S1, S2 and S22 are faint. Spot S13 is prominent, as are three spots above S2, only faintly seen on the 30s pattern (Fig. III (11) D). Two additional peptides are found, one above S14 and one to the left of S13.

To summarise, the fingerprint of the proteins of the large KCl-derived subunit lacks only one spot present on the corresponding EDTA-dissociated subunit pattern; and the 40s subunit contains two peptides additional to the 30s complement. Since the fingerprints are so similar, we have concluded that non-ribosomal protein contamination of the EDTA-dissociated subunit does not present a major problem in interpretation of the two dimensional fingerprints (see also Expt. III A (3)). Loss of one or two spots and faintness of others on the KCl-washed subunit suggests that some ribosomal protein "stripping" does occur, but only one or two proteins are "stripped" completely by the ionic environment used to prepare the subunits.

A number of workers have shown that high salt concentrations "strip" proteins from prokaryotic ribosomes (Spitnik-Elson and Atsmon, 1969; Atsmon, Spitnik-Elson and Elson, 1969). The protein detachment seems to occur sequentially (Lerman et al., 1966; Itoh et al., 1968). Other workers have shown similar protein "stripping" (Reboud et al., 1969; Hamilton et al., 1971; Ford, 1971) in sequential fashion (Clegg and Arnstein, 1970; Grummt and Bielka, 1970) from eukaryotic ribosomes.

However, these workers investigated the protein stripping effects of salts on ribosomal subunits in the presence of low magnesium concentrations (2mM or less). Spitnik-Elson and Atsmon (1969) have shown that, in the presence of high magnesium concentrations (10mM), release of protein is slow, and percentage protein detachment by high monovalent cation concentrations is reduced. Consequently, selective "stripping" of particular 60s subunit proteins by the 0.85M KCl dissociating buffer may be inhibited by the high magnesium concentration (15mM) present in that buffer solution. Indeed, Clegg and Arnstein (1970) propose that magnesium may play a direct role in the binding of at least some proteins to the rRNA thus, presumably, preventing "stripping" of these proteins by KCl.

(2) Fingerprints of LiCl/urea extracted ribosomal subunit proteins.

In the fingerprinting technique described in METHODS, pancreatic ribonuclease is used to digest rRNA and thus release ribosomal proteins from their respective subunits. Because of the risk of proteolysis due to the possible presence of contaminant proteases in the ribonuclease, one experiment was performed in which proteins were extracted from HeLa cell ribosomal subunits by the LiCl/urea method of Leboy et al. (1964). After processing through the two dimensional fingerprinting procedure, the proteins were found to give the pattern shown in Fig. III (12). Subunit fingerprints similar to those derived by ribonuclease digestion have been obtained, although the resolution of the 50s pattern is poorer than that of Fig. III (9). This indicates that no significant degree of proteolysis occurs during ribonuclease treatment of subunits, and consequently, because of its convenience, the ribonuclease method was used throughout the project.

From Expts. III A (2) and (3), and III B (1)b and (2) it was concluded that the fingerprints obtained from EDTA-dissociated subunits are true characterisations of the constituent subunit polypeptides, essentially uncontaminated by cytoplasmic proteins.

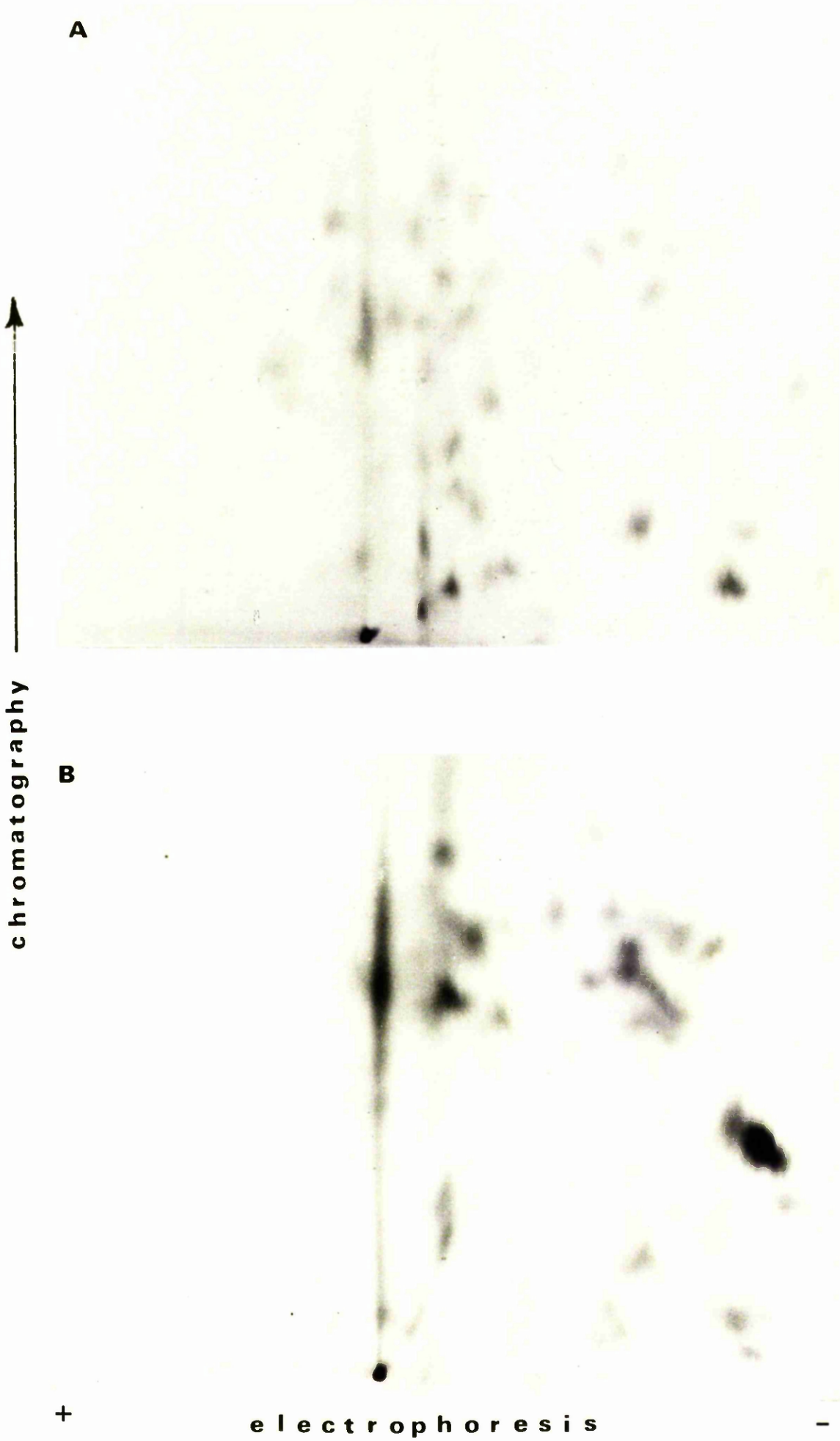
Fig. III (12) Two dimensional peptide fingerprints of LiCl/urea extracted HeLa cell ribosomal subunit proteins

Four cultures of 30 x 10^6 HeLa monolayer cells, each in 200ml of EC 10 containing 0.375mCi L-[^{35}S]methionine (final specific activity = 18mCi/mmol) were incubated for 70 hours at 37°C. The cells were harvested by scraping, ribosomes prepared, dissociated with EDTA, and subunits separated on a 36ml 15-30% sucrose gradient in NEB (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). 50s and 30s peak fractions from the gradient were pooled separately, the particle proteins extracted by the LiCl/urea procedure of Leboy et al. (1964), dialysed overnight against 2 x 5l of distilled water to remove salt and urea, and fingerprinted according to METHODS Section (4) C. Radioactive peptides were located by autoradiography.

Technical details are supplied in the METHODS section.

Panel A: Two dimensional tryptic peptide fingerprint of HeLa 30s ribosomal subunit proteins extracted by LiCl/urea.

Panel B: Two dimensional tryptic peptide fingerprint of HeLa 50s ribosomal subunit proteins extracted by LiCl/urea.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(3) Two dimensional peptide fingerprints of [^{35}S]cystine-labelled HeLa ribosomal proteins.

The possibility of using [^{35}S]cystine as an alternative label to [^{35}S] methionine was also investigated. Autoradiographs of HeLa 50s and 30s ribosomal proteins fingerprinted after [^{35}S]cystine labelling are shown in Fig. III (13). The patterns demonstrate -

(i) Different cysteine containing polypeptide complements for large and small subunits.

(ii) No relatioship to the [^{35}S]methionine fingerprints, suggesting that there is little cysteine-methionine interconversion in the cells (Morgan and Morton, 1957).

(iii) Fair reproducibility in repeat experiments.

However, poor uptake of label over the incubation period rendered [^{35}S] cystine less suitable than [^{35}S]methionine as radioactive marker in these experiments.

(4) Fingerprinting analysis of nucleolar particle proteins.

Section I C describes the evidence which indicates the existence of a precursor-product relationship between 55s nucleolar particles and 50s ribosomal subunits. Evidence is also presented to suggest a similar relationship between the RNA's of 80s nucleolar particles and 30s and 50s ribosomal subunits; but, although preliminary experiments have demonstrated some 50s proteins on the 80s particle (Maden and Warner, unpublished), polypeptides from the small subunit have not been extracted from that source. It was not clear whether they do not occur on the 80s precursor or, conversely, whether their presence on that precursor is masked by large subunit proteins. The experiment described in the legend of Fig. III (14) was performed to investigate this problem. The 50s/30s mixture reconstruction from Fig. III (10) has been included to facilitate determination of the subunit of origin of each polypeptide on the 55s and 80s "maps". Several points arise from this experiment:-

(i) The 55s pattern resembles closely a 50s ribosomal protein fingerprint,

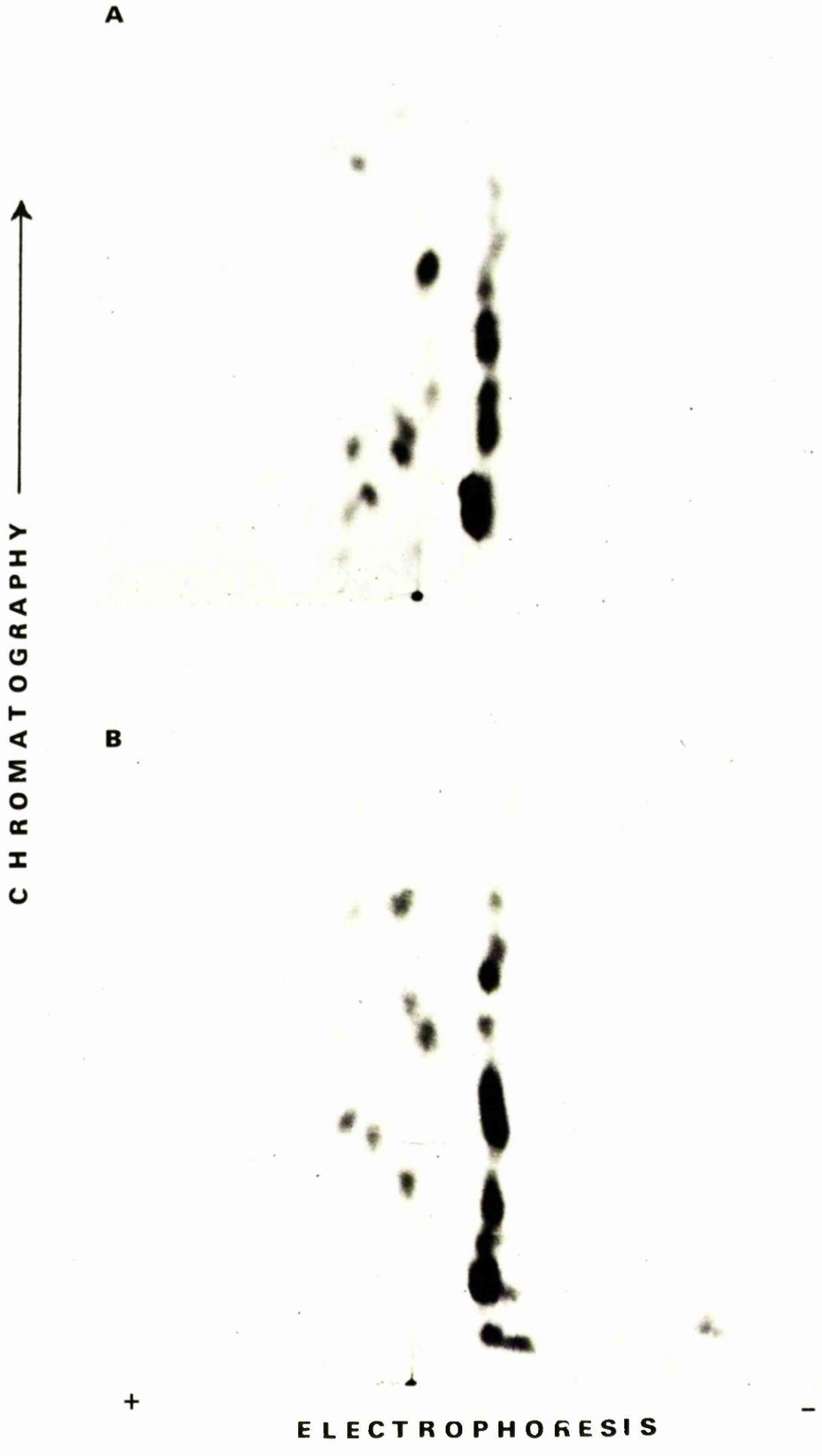
Fig. III (13) Two dimensional peptide fingerprint of L-[^{35}S] cystine-labelled HeLa ribosomal subunits

25 x 10^6 HeLa monolayer cells were incubated for 68 hours at 37°C in 200ml EC 10 containing 1mCi L-[^{35}S]cystine (final specific activity = 40mCi/mmol). The cells were harvested by trypsinisation, ribosomes prepared, dissociated into subunits with EDTA, and the subunits separated in a 36ml 15-30% sucrose gradient in NEB (17 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Subunit peak fractions were pooled separately and their proteins extracted and fingerprinted in two dimensions according to METHODS Section (4) C. In this experiment, tryptic digestion of the proteins was for 36 hours.

All experimental details are provided in METHODS.

Panel A: Two dimensional cystine labelled tryptic peptide fingerprint of HeLa 50s ribosomal subunit proteins.

Panel B: Two dimensional cystine labelled tryptic peptide fingerprint of HeLa 30s ribosomal subunit proteins.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substantiating the relationship between these two particles, and the role of the nucleolus in ribosome assembly. There are a few 50s peptides absent from the 55s particle (L16, L17) and this may be significant (Warner and Soeiro, 1967; Expts. III B (5) and (6)). Typical 30s spots are not found on the 55s fingerprint.

(ii) The 80s fingerprint exhibits all spots found on the 55s "map", and also a small number of additional peptides. These spots (S3, S9, S14 (faint), S17, S18, S20, S21, S37 - see Fig. III (10) for code) have the same electrophoretic and chromatographic mobilities as certain spots characterised in Fig. III (10) A as typical 30s peptides. However, by visual observation of spot intensities, it can be seen that the 30s peptides on the 80s particle are represented in lower molar amounts than the 55s peptides.

(iii) Repeated attempts to isolate 80s particles containing all of the characteristic 30s peptides have been unsuccessful.

(iv) Since it was known that the 80s particle is unstable on centrifugation (Expt. III A (4)), material from the top of a [^{35}S]methionine-labelled nucleolar particle preparative gradient was fingerprinted according to the procedure detailed in METHODS. The result is shown in Fig. III (14) D. Some spots have mobilities which suggest the presence of 50s ribosomal material, but the majority of heavily labelled peptides can be related to spots typically of small subunit origin (S3, S4, S9, S14, S20, S22 (faint), S37). Although the absence of marker peptides on the fingerprints makes absolute spot identification impossible, the results suggest that, during isolation of the 80s particle by centrifugation, 30s proteins are stripped from the particle and appear, because of their low buoyant density, at the top of the sucrose gradient. Alternatively, many of the 30s peptides may never have been associated with 80s particles, but may have existed free in the nucleolus. The implications of these suggestions will be discussed later.

Fig. III (14) Two dimensional fingerprints of HeLa 80s and 55s nucleolar particle proteins

1.2×10^8 HeLa S3 cells were washed in methionine free Joklik modified EC 10 and were then labelled for 30 minutes at 37°C in 50ml of the same medium containing 1mCi L-[^{35}S]methionine (final specific activity = 17Ci/mmol). The cells were harvested, nucleolar particles prepared and separated in a 36ml 15-30% sucrose gradient in NEB (16 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The gradient was collected into approximately 30 fractions, the 80s and 55s peak fractions pooled, the particle proteins extracted, and fingerprinted according to METHODS Section (4) C. Radioactive peptides were located by autoradiography.

All technical details are provided in METHODS.

Panel A: Two dimensional tryptic peptide fingerprint of 80s nucleolar particle proteins.

Panel B: Two dimensional tryptic peptide fingerprint of 55s nucleolar particle proteins.

Panel C: Equimolar 50s/30s HeLa ribosomal subunit protein fingerprint reconstruction from Fig. III (10), provided for comparative purposes.

Panel D: Two dimensional tryptic peptide fingerprint of material isolated from the top of the nucleolar particle preparative gradient.

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(5) "Pulse-chase" labelling of nucleolar ribonucleoprotein particles and cytoplasmic ribosomal subunits.

The experiment described in the legend of Fig. III (15) was performed to substantiate the findings of Expt. III B (4) and to confirm that 50s and 30s ribosomal subunit proteins derive (at least in part) from the 80s particle protein complement. Peptide fingerprints from nucleolar and ribosomal particles are shown in Fig. III (16). The experiment provides the following information:-

(i) During a 30 minute "pulse", [^{35}S]methionine is incorporated rapidly into nucleolar particles, and the subsequent three hour "chase" with a 3,300 fold excess of unlabelled methionine removes effectively most radioactivity from these particles (Fig. III (15)).

(ii) The reverse is true in the case of the cytoplasmic ribosomal subunits. After a 30 minute "pulse" with [^{35}S]methionine, the specific activity is low, but rises slowly during the three hour "chase" to reach a maximum value (Fig. III (15)).

These observations imply that there may exist a precursor-product relationship between nucleolar particles and cytoplasmic ribosomal subunits.

(iii) Fig. III (16) confirms this relationship between nucleolar particles and ribosomes.

After a 30 minute "pulse"

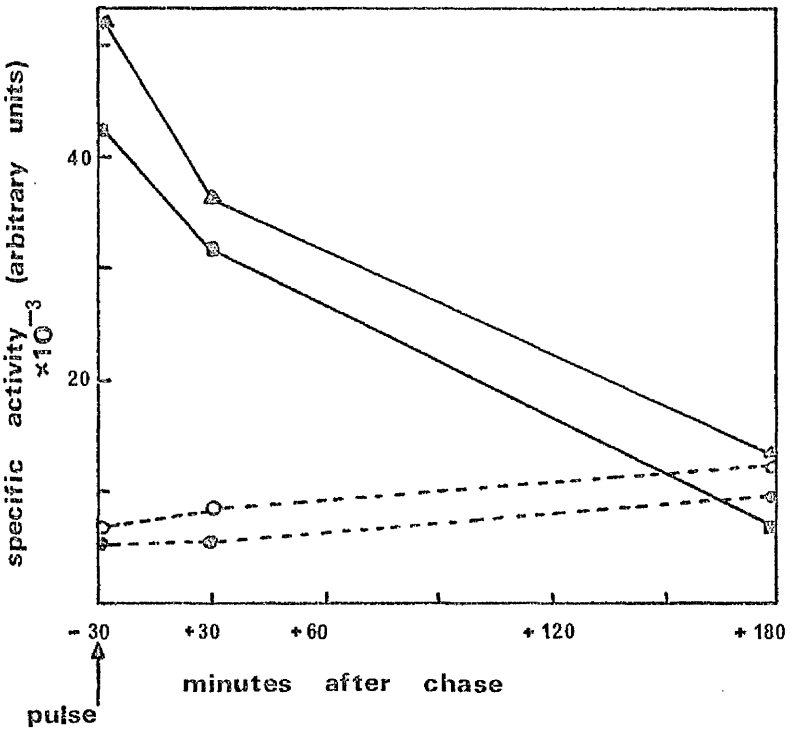
Most characteristic 50s peptides are found on the 80s and 55s fingerprints, and the 80s pattern also contains a few 30s peptides (S3, S14, S20, S21, S22).

The 30s cytoplasmic subunit fingerprint resembles an equilibrium-labelled 30s pattern, although a few spots (S9, S18, S26, S32) are indistinct. This confirms that maturation of the small subunit is rapid (Girard et al., 1965) and suggests that the cell "pool" of 30s ribosomal proteins is small and equilibrates rapidly with newly synthesised [^{35}S]methionine-labelled ribosomal proteins (see review by Maden, 1971).

The 50s fingerprint does not resemble the equilibrium labelled large

Fig. III (15) "Pulse-chase" labelling of HeLa cell nucleolar particles and cytoplasmic ribosomes

3×10^8 HeLa S3 cells were washed in methionine-free Joklik modified EC 10 and suspended at 37°C in 160ml of the same medium, in the presence of 2mCi of L-[^{35}S]methionine (final specific activity = 18Ci/mmol). After 30 minutes, a 50ml aliquot of cells was removed and chilled immediately, and the radioactivity in the remaining culture was "chased" by addition of a 500 fold excess of unlabelled methionine. A second 50ml aliquot of cells was removed and chilled after 30 minutes "chase" and a third aliquot after a further $2\frac{1}{2}$ hours "chase". Immediately after sampling, each cell aliquot was used to prepare nucleolar particles and ribosomes (EDTA dissociated), according to the procedures in METHODS. The nucleolar particles and subunits, respectively, were separated on 16ml and 36ml 15-30% sucrose gradients in NEB (17 hours, 75,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Each gradient was collected into approximately 30 fractions while the absorbance at 260mμ was monitored continuously on a Gilford 240 spectrophotometer. Peak fractions of each particle species were pooled and total peak radioactivity determined by counting aliquots from each species in a Nuclear Chicago low background gas flow counter. An arbitrary specific activity for each particle species was calculated by relating total radioactivity in each peak to the area under its 260mμ absorbance profile. The changes in specific activity throughout the period of the "chase" are shown opposite.


specific activity (arbitrary units) ×10⁻³
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20
- 30
+30
+60
+120
+180
pulse
minutes after chase
80
50
55
30

Fig. III (16) Fingerprints of nucleolar particle and ribosomal proteins after "pulse-chase" labelling with L-[^{35}S]methionine.

The proteins of the nucleolar particles and ribosomal subunits prepared as described in the legend of Fig. III (15) were analysed by the two dimensional fingerprinting technique detailed in METHODS Section (4) C. Radioactive spots were located by autoradiography.

Panel A: Two dimensional tryptic fingerprint of 80s proteins after a 30 minute "pulse" with L-[^{35}S]methionine.

Panel B: Two dimensional tryptic fingerprint of 80s proteins "pulsed" for 30 minutes with L-[^{35}S]methionine and "chased" for 30 minutes with unlabelled methionine.

Panel C: Two dimensional tryptic fingerprint of 55s proteins after a 30 minute "pulse" with L-[^{35}S]methionine.

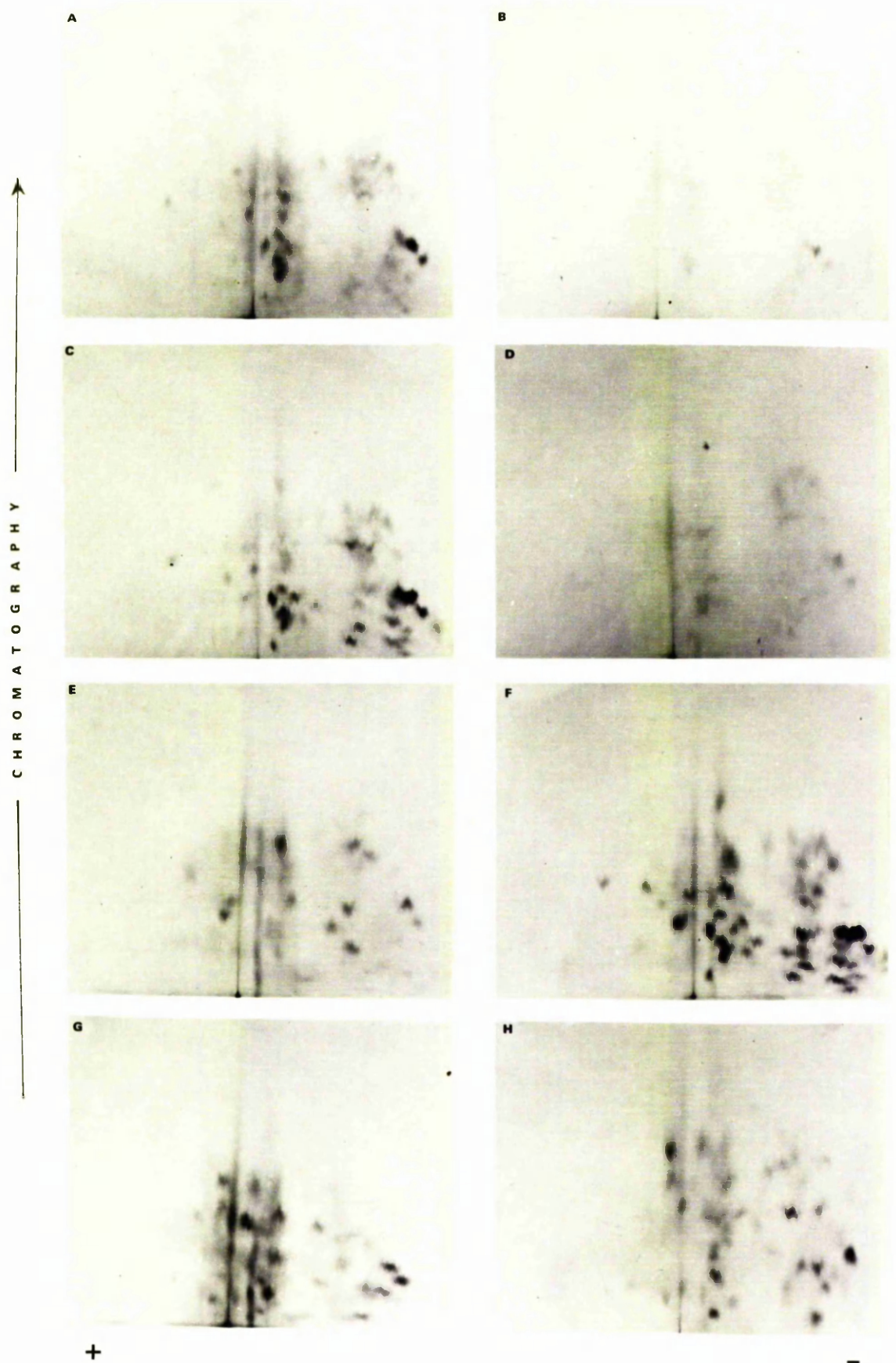
Panel D: Two dimensional tryptic fingerprint of 55s proteins "pulsed" for 30 minutes with L-[^{35}S]methionine and "chased" for 3 hours with unlabelled methionine.

Panel E: Two dimensional tryptic fingerprint of 50s proteins after a 30 minute "pulse" with L-[^{35}S]methionine.

Panel F: Two dimensional tryptic fingerprint of 50s proteins "pulsed" for 30 minutes with L-[^{35}S]methionine, and "chased" for 3 hours with unlabelled methionine.

Panel G: Two dimensional tryptic fingerprint of 30s proteins after a 30 minute "pulse" with L-[^{35}S]methionine.

Panel H: Two dimensional tryptic fingerprint of 30s proteins "pulsed" for 30 minutes with L-[^{35}S]methionine and "chased" for 3 hours with unlabelled methionine.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subunit pattern (Fig. III (9) A and (10) B), but consists of approximately 9-12 prominent peptides surrounded by a number of very faint spots in the characteristic equilibrium-labelled 50s distribution. The presence of these faint peptides permits identification of some of the prominent 50s spots, which were found to be L4,L8, L21, L22, L31, L32. A few of the prominent "pulse-labelled" peptides were present in relatively small quantities on the mature subunit fingerprint (e.g. L21, L22).

After a 30 minute "chase" (N.B. only the 80s 30 minute "chase" fingerprint appears in Fig. III (16)).

The 30s peptides originally found on the 80s fingerprint cannot now be identified, and the 50s peptides on the 80s and 55s "maps", while still present, are becoming indistinct. This confirms that maturation of the nucleolar particles is rapid (see Fig. III (15)), and suggests that any 30s proteins which associate with 80s material have a shorter transit time through nucleolar particles than do 50s peptides. Alternatively, it may indicate that the 30s subunit protein pool is smaller than that of the large subunit (see Maden and Vaughan, 1968; Willems et al., 1969; Craig and Perry, 1970; Maden, 1971).

The 30s ribosomal subunit fingerprint bears all the characteristic features of an equilibrium-labelled 30s peptide pattern, while the 50s fingerprint is unchanged from the 30 minute "pulse" pattern.

From these findings it has been concluded that all small ribosomal subunit proteins are labelled in the cytoplasm within 30-60 minutes, while complete labelling of the cytoplasmic large subunit protein set takes more than 60 minutes.

After a three hour "chase"

The label in the 80s particles has almost disappeared (insufficient for fingerprinting), and the peptides on the 55s fingerprint are faint.

The 30s cytoplasmic fingerprint remains complete, and the 50s fingerprint has lost the unusual features originally described and now is identifiable

as a characteristic equilibrium-labelled pattern (cf. Fig. III (16) F and III (9) A).

The results from this experiment confirm the data of Expt. III B.(4), and indicate the existence of a precursor-product relationship between the proteins of the 80s particle and those of the cytoplasmic ribosomal subunits (50s and 30s together). Moreover, a similar relationship is apparent between 55s and 50s particle proteins, in confirmation of earlier work by Warner and Soeiro (1967). Again, however, a full 30s protein complement has not been isolated from the 80s particle.

(6) Cytoplasmic "exchangeable" proteins and the large ribosomal subunit: The effects of actinomycin D and toyocamycin on ribosomal protein labelling patterns.

In HeLa cells, newly labelled small ribosomal subunits are found in the cytoplasm within 20-30 minutes of incubation with radioactive RNA or protein precursors, while labelled 50s subunits are not apparent before approximately 60 minutes have elapsed (Girard et al., 1965; Expt. III B (5)). Therefore, the appearance of prominently labelled peptides on the large ribosomal subunit after a 30 minute "pulse" (Fig. III (16) E) was unexpected. However, this result did agree with earlier findings of Warner (1966) and Warner and Soeiro (1967) who provided evidence to suggest that interchange of a (few) protein (s) occurred between the cytoplasmic protein "pool" and the mature large ribosomal subunit.

The experiments described in Fig. III (17) confirmed and extended their observations. Actinomycin D induced inhibition of rpreRNA transcription and transport from the nucleolus (Penman, 1966), or toyocamycin mediated inhibition of rpreRNA maturation (Tavitian et al., 1968, 1969; Sverak et al., 1969) effectively prevents new ribosome formation. However, under these conditions, existing large ribosomal subunits in the cytoplasm continue to incorporate [^{35}S]methionine-labelled proteins into their structure. These proteins, on fingerprinting, present a strikingly similar pattern to that derived from 50s subunits "pulsed" with [^{35}S]

Fig. III (17) Fingerprinting studies of HeLa cell ribosomes labelled in the presence of actinomycin D or toyocamycin

(A) Labelling in the presence of actinomycin D

Approximately 10^8 HeLa monolayer cells were preincubated for 60 minutes at 37°C in 50ml EC 10 containing 4µg/ml of actinomycin D. The medium was changed to methionine-free EC 10 containing 4µg/ml actinomycin D and, after addition of 1mCi L-[^{35}S]methionine (17Ci/mmol), incubation was continued for 60 minutes. The label was then "chased" for 5 minutes with an 800 fold excess of unlabelled methionine, the cells harvested by scraping, and ribosomes prepared as described in METHODS. The ribosomes were dissociated with EDTA and separated into subunits in a 36ml 15-30% sucrose gradient in NEB (17 hours, 80,000g, +4°C in a Beckman L2 ultra-centrifuge with SW 27 rotor). 50s and 30s peak fractions were pooled and the subunit proteins fingerprinted by the procedure detailed in METHODS Section (4) C.

(B) Labelling in the presence of toyocamycin

The experiment described in (A) was repeated, actinomycin D being replaced by 0.2µg/ml of toyocamycin as inhibitor of ribosome maturation. Again, ribosomal subunits were separated on sucrose gradients and fingerprinted as described above and in METHODS.

Radioactive peptides were located by autoradiography.

Panel A: Two dimensional peptide fingerprint of HeLa 50s ribosomal subunit proteins labelled in the presence of actinomycin D.

Panel B: Two dimensional peptide fingerprint of HeLa 50s ribosomal subunit proteins labelled in the presence of toyocamycin.

Panel C: Two dimensional peptide fingerprint of HeLa 30s ribosomal subunit proteins labelled in the presence of actinomycin D. The pattern obtained by labelling in the presence of toyocamycin was identical.


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methionine for 30 minutes (cf. Figs. III (17)A and (16)E). It appears therefore, that, in confirmation of the findings of Warner and Soeiro (1967), there exists an interchange of cytoplasmic proteins with large ribosomal subunits in the absence of new ribosome formation. There is no detectable protein exchange with the small ribosomal subunit (Fig. III (17)C).

Although it has been shown that contamination of the ribosome with non-ribosomal proteins is unlikely during the subunit isolation procedure (Expt. III A. (3)), cytoplasmic "exchangeable" proteins may bind, during extraction, to the 50s subunit. To examine this possibility, the experiment described in Fig. III (17) was repeated using actinomycin D as ribosome maturation inhibitor. In this case, however, the ribosomes were dissociated and separated by the 0.85M KCl procedure of Martin and Wool (1968), which is known to be effective in stripping non-ribosomal proteins from the particles (Spirin and Gavrilova, 1969). The resulting fingerprints (not shown) demonstrate that these peptides found on the large subunit in Fig. III (17)A persist after salt-washing, suggesting that the exchangeable proteins are not adventitious, non-ribosomal contaminants.

It is noteworthy that after [^{35}S]methionine-labelling of ribosomal proteins for 60 minutes in the presence of actinomycin D or toyocamycin, the ratio of radioactivity in the large/small subunit is 2.01 and 1.74 respectively while it is 1.2 in control ribosome preparations labelled in the absence of the drug. The fact that some label appears in the small subunit in the presence of either drug presumably indicates residual maturation of nuclear 30s material into cytoplasmic 30s subunits.

The above experimental observations suggest that, in the absence of new ribosome formation, there is specific addition of proteins to pre-existing cytoplasmic 50s ribosomal subunits. The added proteins resist the "stripping" effects of a salt-wash. The small ribosomal subunit does not

participate in this cytoplasmic particle-protein interaction.

Finally, Manteuil et al. (1970) have reported, on the basis of gel analysis from an experiment of similar design to that of Fig. III (17), that the labelled proteins which associate with ribosome monomers in the presence of actinomycin D differ from those which are added to toyocamycin-treated ribosomes. The results presented here do not support this view, but indicate that the proteins added to the large subunit are independent of the drug used to block ribosome maturation (cf. Figs. III (17)A and B). The reason for this discrepancy is not clear.

Section III B - Summary

This section describes two dimensional fingerprinting studies which were performed on the proteins derived from the HeLa ribosomal subunits and nucleolar particles characterised in Section III A. The salient data are summarised below.

(1) Proteins from large (50s) and small (30s) EDTA-dissociated ribosomal subunits yielded different and reproducible [^{35}S]methionine-labelled peptide autoradiographs. Radioactive peptides characteristic of each subunit were located on their respective fingerprint. Peptide patterns of proteins isolated from 0.85M KCl-dissociated ribosomal subunits were essentially similar to their EDTA-derived counterpart, indicating that the protein content of the particles was not influenced by the method of subunit preparation. Likewise, LiCl/urea extracted subunit proteins gave identical fingerprints to those obtained by the RNase procedure described in METHODS, again confirming that the peptide maps obtained are not an artefact of the protein extraction procedure.

(2) [^{35}S]cystine-labelled ribosomal proteins provided simple and characteristic "maps" for each subunit, but poor uptake of label into the cells rendered this amino acid less suitable than [^{35}S]methionine as a radioactive marker in this study.

(3) Nucleolar 55s ribonucleoprotein particles from cells labelled for short periods yielded peptide "maps" closely similar to those derived from

equilibrium-labelled 50s ribosomal subunits. The labelled peptides could be "chased" from 55s particles to 50s ribosomal subunits on further incubation of the cells with an excess of unlabelled methionine. However, after a short "pulse" with [^{35}S]methionine, 50s ribosomes were found to contain a small number of radioactive peptides on fingerprinting. These peptides also became labelled when ribosome maturation was inhibited by actinomycin D or toyocamycin. At least some of these peptides were not present in fingerprints of 55s particle proteins.

(4) Radioactivity appeared more rapidly in 30s than in 50s proteins, but a few 30s peptides became labelled more slowly than the remainder. Actinomycin D and toyocamycin effectively blocked all labelling of 30s proteins.

(5) Nucleolar 80s ribonucleoprotein particles yielded a predominantly 55s-like peptide "map", although some weakly but significantly labelled 30s peptides were apparent on the 80s fingerprint. Protein from the tops of sucrose gradients used for preparing nucleolar particles was also found to contain some 30s and a few 50s peptides on fingerprinting. The low yield of 30s proteins on the 80s particle was confirmed by several experiments. One possible cause of such a phenomenon was that gross contamination of 80s particles with 55s material may have masked the presence of 30s peptides in those particles. Therefore, in the light of the above findings, and although it had been shown previously that 80s nucleolar particulate material isolated on sucrose gradients was 70-80% pure (Expt. III A (4), it was decided to re-examine the purity of both 80s and 55s ribonucleoprotein species. This was done by RNA analysis using

(1) Sucrose gradient centrifugation, and

(2) Two dimensional RNA fingerprinting.

The results are described in the next section.

SECTION III C - Analysis of nucleolar particle RNA.

As already indicated, Warner and Soeiro (1967) have shown that 80s and 55s nucleolar particles contain 45s and 32s RNA precursors of mature 28s and

18s rRNA. The precursor RNA was re-examined in the present study, first to confirm that the particles investigated here were identical to those described by Warner and Soeiro (1967), and secondly, to assess, by a method independent of that delineated in Expt. III A (4), the degree of cross-contamination of 80s with 55s material.

The nucleolar particle RNA was analysed by

(1) Sucrose gradient centrifugation and

(2) Two dimensional RNA fingerprinting

(1) Sucrose gradient centrifugation of RNA extracted from nucleolar particles

Cells were "pulse" labelled with [^{3}H]uridine, nucleolar particles isolated on sucrose gradients, the RNA extracted from the particles, and re-centrifuged on gradients as described in the legend of Fig. III (18). The results show that the major RNA peak isolated from the 80s particle has a sedimentation coefficient of 45s. Contaminant material sedimenting at lower S values (indicated by a shoulder on the radioactivity profile - Fig. III (18)B) amounts to approximately 32% of the radioactivity applied to the gradient.

The main peak of radioactivity on recentrifugation of RNA extracted from the 55s particles (Fig. III (18)C) has a sedimentation coefficient of approximately 32s, but also shows some contamination with more rapidly (45-41s)and slowly sedimenting material. The latter presumably is derived by 32s RNA degradation. The former, 45s contaminant, is difficult to eliminate from 32s rpreRNA (Salim et al., 1970), but can be eradicated by blocking new 45s RNA synthesis with actinomycin D and allowing degradation of existing 45s material in vivo before RNA extraction from the nucleolus (Maden et al., 1972).

The above experimental data imply that 80s material isolated from the sucrose gradient of Fig. III (18)A is approximately 70% pure. The following experiment, using the RNA fingerprinting technique originally described by Sanger et al. (1965) was devised to provide an independent

Fig. III (18) Isolation and centrifugation of RNA from HeLa cell 80s and 55s nucleolar particles

3×10^8 HeLa S3 cells were labelled for 45 minutes at 37°C with 500μCi [^{3}H]uridine (5Ci/mmol) in 400ml Joklik modified EC 10. The cells were harvested and nucleolar particles prepared according to METHODS. The 80s and 55s particles were separated in a 36ml 15-30% sucrose gradient in NEB (16 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The gradient was collected into approximately 30 fractions with simultaneous monitoring of the absorbance at 260mμ in a Gilford 240 spectrophotometer. To the pooled 80s and 55s peak fractions (Fig. III (18) A) were added NaCl to 0.2M, SDS to 0.2% and 2.5 volumes of absolute ethanol. After standing at -10°C for 6 hours, the faintly opalescent RNA precipitate was collected by centrifugation (30 minutes, 5,000g, -10°C), redissolved in 1.0ml of LETS containing unlabelled "marker" HeLa 28s and 18s rRNA, and reapplied to a 36ml 15-30% sucrose gradient in LETS. Recentrifugation (16 hours, 62,000g, +20°C in a Beckman L2 ultracentrifuge with SW 27 rotor) was followed by collection of the gradients into approximately 30 fractions. Each fraction was made 10% with TCA, acid insoluble material collected on membrane filters and radioactivity assayed in a Packard Tricarb spectrometer using toluene based scintillant.

Technical details are found in METHODS.

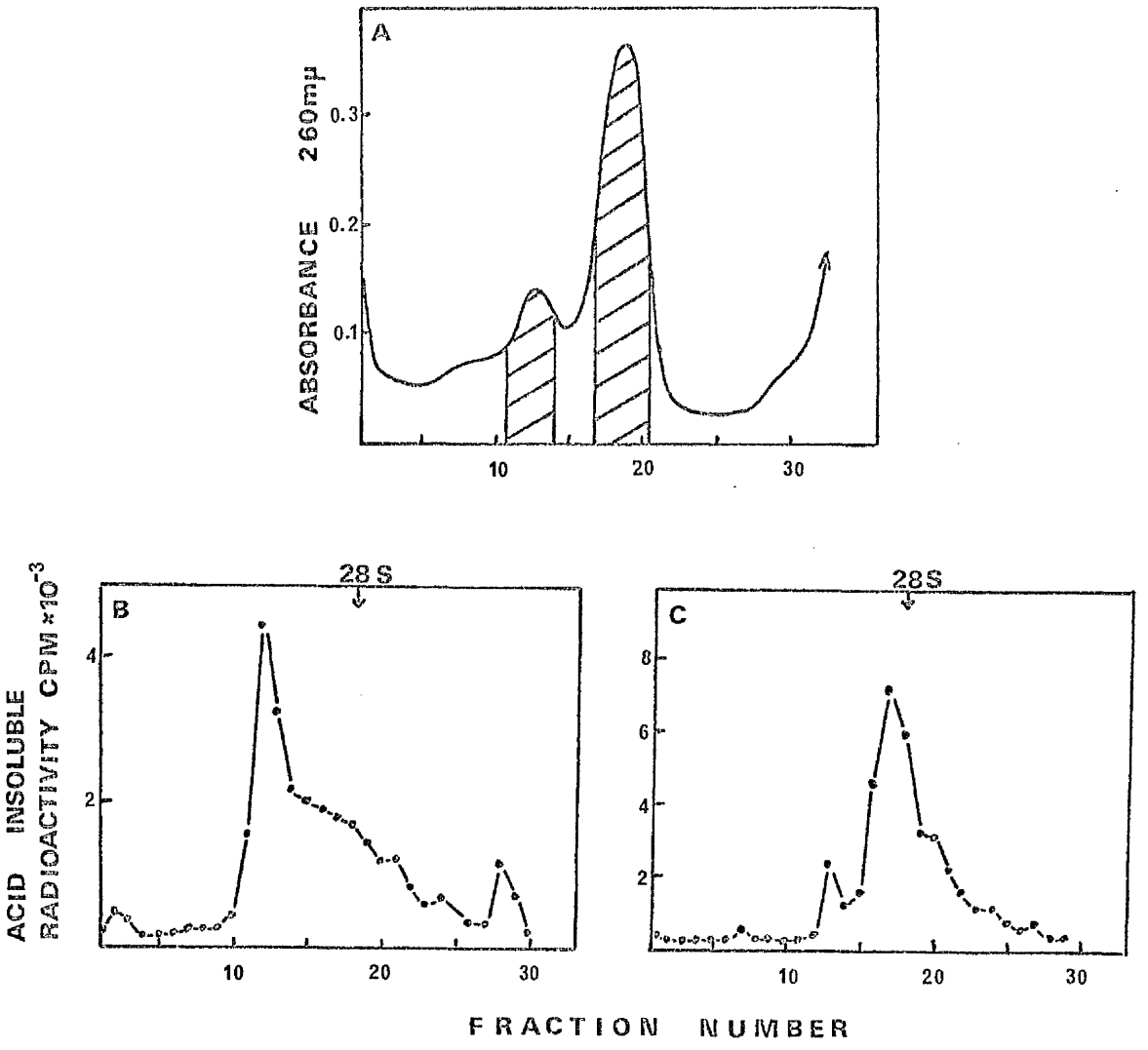
Panel A: Absorbance profile of nucleolar particles, showing pooled peaks taken.

Panel B: Radioactivity profile of RNA extracted from 80s nucleolar particles.

Panel C: Radioactivity profile of RNA extracted from 55s nucleolar particles.

The arrows indicate the position of 28s unlabelled "marker" HeLa rRNA.

Sedimentation is from right to left.


A
ABSORBANCE 260mμ
0.3
0.2
0.1
10
20
30
28S
B
C
ACID INSOLUBLE RADIOACTIVITY CPM ×10⁻³
4
2
8
6
4
2
10
20
30
FRACTION NUMBER

check on the degree of contamination of the nucleolar particles and to determine whether the 45s and 32s RNA's isolated in Expt. III C.(1) contain unique RNA sequences ultimately found in 28s and 18s rRNA.

(2) Fingerprinting studies of nucleolar particle RNA

Salim et al. (1970) have adopted the RNA fractionation technique of Sanger et al. (1965) to show that [methyl-^{14}C]methionine-labelled 45s RNA contains virtually all of the methylated oligonucleotide sequences found in 28s and 18s rRNA (one "late" methylation step on the 18s rRNA seems to occur after its transfer to the cytoplasm (Zimmerman, 1968; Maden et al., 1972)). Their findings confirm earlier similar suggestions by other workers (Girard et al., 1964; Greenberg and Penman, 1966; Zimmerman and Holler, 1967; Wagner et al., 1967; Weinberg and Penman, 1970). Likewise, Maden et al. (1972) have demonstrated that 32s nucleolar RNA contains methylated sequences found on 28s rRNA, but not on 18s rRNA. These data are in agreement with the rpreRNA maturation scheme proposed by Weinberg and Penman (1970) - Fig. I (1). Fingerprinting of [methyl-^{14}C]methionine-labelled nucleolar RNA extracted from nucleolar particles to assay for relative amounts of "28s" and "18s" oligonucleotides should provide an independent means of determining the purity of these particles.

With the above end in view, HeLa cells were labelled with L-[methyl-^{14}C] methionine for 2.5 hours in the presence of adenosine, guanosine and sodium formate (Winocour et al., 1965) to inhibit purine ring labelling, and nucleolar particles were isolated and separated on sucrose gradients as described in METHODS. RNA was extracted from the particle peak fractions and fingerprinted. The final yield of radioactive rpreRNA was low and long exposure times (6 months) were needed to obtain the results shown in Fig. III (19). A reconstruction of a fingerprint of an equimolar mixture of 28s and 18s HeLa rRNA (from Maden et al., 1972) has been included (Fig. III (19)B) to demonstrate that the RNA derived from the 80s particle contains almost all spots found in fingerprints of a mixture of

Fig. III (19) Fingerprints of RNA derived from HeLa 80s and 55s nucleolar particles.

10^8 HeLa S3 cells were incubated for 45 minutes in 50ml methionine-free Joklik modified EC 10 containing 10mM sodium formate, 2×10^{-5}M adenosine and guanosine and 100μCi L-[methyl-^{14}C] methionine (final specific activity = 56mCi/mmol). The cells were harvested, nucleolar particles prepared and separated by overnight centrifugation in a 36ml 15-30% sucrose gradient in NEB (16 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). 80s and 55s particle peak fractions were pooled and the RNA extracted from the particles and fingerprinted as described in METHODS Section (4)d.

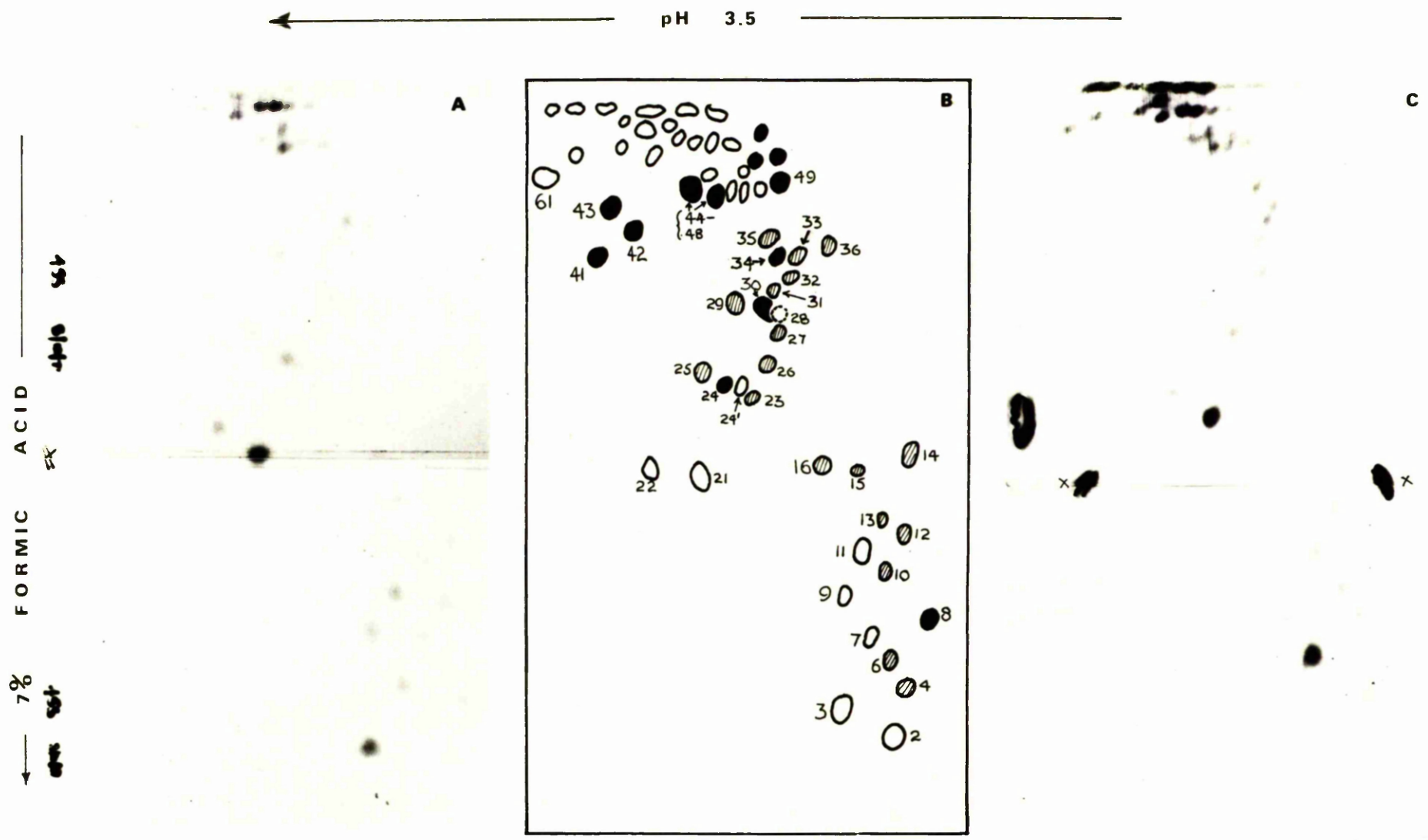
Radioactive methylated oligonucleotides were detected by autoradiography.

All technical procedures are detailed in METHODS.

Panel A: Two dimensional fingerprint of RNA extracted from HeLa 80s nucleolar particles.

Panel B: Two dimensional fingerprint of an equimolar mixture of HeLa 28s and 18s RNA showing 28s [ZZZ] and 18s [▒▒] specific oligonucleotides (from Maden et al., 1972).

Panel C: Two dimensional fingerprint of RNA extracted from 55s HeLa nucleolar particles. Areas marked "X" are artefacts on the autoradiograph.


pH 3.5
FORMIC ACID
7%
A
B
C
61
43
42
41
49
44-48
35
33
36
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32
30
31
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18s and 28s rRNA while that from the 55s particle contains characteristic 28s spots (with trace contamination from 45s RNA appearing as faint 18s spots - numbers 41, 42, 43). Maden has shown (personal communication) that the ratio of total radioactivity in 18s spots (8, 24, 41, 42, 43 - Fig. III (19)B) to radioactivity in 28s spots (4, 6, 10, 12, 25, 26, 29) in a pure 45s fingerprint is approximately 0.860. The ratio of radioactivity in the same spots of the 80s-extracted RNA fingerprinted in Fig. III (19)A is 0.690, indicating the presence of 24.6% contaminant RNA from the 55s particle.

SECTION III C - SUMMARY

RNA's derived from 80s and 55s nucleolar particles contain the methylated oligonucleotide sequences found on nucleolar 45s and 32s RNA respectively (cf. Fig. III (19) with Maden et al., 1972). Moreover, their sedimentation coefficients allow them to be classified as "45s" and "32s" RNA.

The degree of cross-contamination present in isolated nucleolar particles has been assessed using three independent techniques. The 80s particle, after glutaraldehyde fixation and recentrifugation was found to be approximately 73% pure (Expt. III A (4)). Sucrose gradient analysis of RNA extracted from the 80s particle indicated that the particle was 68% pure (Expt. III C.(1)); and fingerprinting studies of similarly derived [Methyl-^{14}C]methionine-labelled RNA showed that its nucleolar particle of origin was 75% pure.

Isolated 55s nucleolar particles appear to be minimally contaminated with 80s material ($<$10%).

Cross-contamination of 80s particles with 55s material will undoubtedly alter the molar ratios of 50s and 30s proteins potentially present on these particles and influence the interpretation of two dimensional fingerprint studies of 80s nucleolar particle proteins. The experiments presented in Section D describe a number of methods used in an attempt to minimise contamination of 80s particles during their isolation and to

improve the yield of 30s proteins potentially present on these particles.

SECTION III D - Procedures used to increase the yield of 80s nucleolar particles and reduce cross-contamination by 55s material

A number of points are evident from experiments III B. (4) and (5), and from other pilot studies:-

(i) 80s nucleolar particles containing all 30s ribosomal proteins as detected by the two dimensional fingerprinting technique have not been isolated.

(ii) The yield of 80s particles on nucleolar extraction is low. Pilot experiments have shown that 80s material extracted as in METHODS contains only 35% of the total 45s nucleolar RNA.

(iii) The ratio of 80s/55s extracted nucleolar material could not be increased by modifying the method of particle isolation.

(iv) 80s material isolated on sucrose gradients is contaminated with 55s particles (Expts. III A. (4); III C. (1) and (2)). This contamination is in the region of 25-30% and presumably arises because of

(a) Preponderance of 55s material over 80s

(b) Proximity of 55s and 80s peaks on sucrose gradients.

The following techniques were examined in an attempt to reduce 55s contamination and increase the absolute yield of 80s material extracted from HeLa cell nucleoli.

(1) Short "pulse-labelling" of cells followed by nucleolar particle extraction.

The following experiment was performed to investigate the speculation that, like uridine-labelling of rpreRNA, a short "pulse" of [^{3}H]leucine should label selectively the larger nucleolar ribonucleoprotein particle and so provide specific enrichment for radioactive 80s particle proteins. 5×10^{8} HeLa cells were grown in 160ml of leucine-free Joklik modified EC10 in the presence of L-[^{3}H]leucine and [^{14}C]uridine as described in

the legend of Fig. III (20). 50ml aliquots were sampled from the culture at times 15, 30 and 45 minutes after labelling, and used to prepare nucleolar particles and ribosomes (by EDTA dissociation) as described in METHODS. Particle separation on sucrose gradients was followed by TCA precipitation and double label scintillation counting on membrane filters. The results, shown in Figs. III (20) and (21) demonstrate the following:-

(i) Nucleolar RNA labelling

Labelling of the RNA moieties of the 80s and 55s nucleolar particles follows the pattern described by Penman (1966) and Penman et al. (1966), confirming the relationship between the 45s and 32s RNA species pertaining to these particles.

After a 15 minute "pulse", label is found in the 45s RNA of 80s particles. By 30 minutes, labelling is shared approximately equally between the 80s and 55s particles (i.e. between 45s and 32s RNA); and after 45 minutes of labelling, 32s RNA (in 55s particles) is the predominant radioactive species. The shoulder of radioactivity, seen in several experiments to sediment at 70s after a 15 minute [^{14}C]uridine "pulse" (Fig. III (20) B inset), is of interest, and may indicate the presence of an intermediate particle (possibly containing 41s RNA - see Weinberg and Penman, 1970) in the maturation of 80s to 55s material, present in such small amount as to be undetected by UV spectrophotometry.

(ii) Nucleolar protein labelling

In contradistinction to the RNA labelling pattern, after 15 minutes of [^{3}H]leucine labelling, radioactivity is found both on 80s and 55s particles (Fig. III (20)A), although at this time there is slight enrichment for radioactive 80s material (relative to the 55s particle, there is 10% more label in the 80s material at 15 minutes than at 45 minutes - Fig. III (21)). This pattern of nucleolar particle protein labelling does not lend itself to a straightforward explanation. Labelled proteins appear very rapidly on both 80s and 55s particles, and although there is evidence for a limited flow of protein radioactivity from the 80s to the 55s particle

Fig III (20) Kinetics of appearance of [^{3}H]-leucine and [^{14}C]-uridine in HeLa cell nucleolar particles and cytoplasmic ribosomes.

5 x 10^{8} HeLa S3 cells, previously washed in leucine-free Joklik modified EC 10 medium, were suspended at 37°C in 160ml of the same medium to which was added 500μCi L-[^{3}H] leucine (final specific activity 19Ci/mmol) and 5μCi[^{14}C] uridine (60mCi/mmol). 50ml aliquots were sampled at 15, 30 and 45 minutes after addition of label, were chilled immediately in ice and used to prepare nucleolar particles and EDTA-dissociated ribosomal subunits. The particles were separated in 30ml 15-30% sucrose gradients in NEB (16 hours, 75,000g, +4°C in a Beckman L2 ultracentrifuge with SW 25.1 rotor). The gradients were collected into approximately 30 fractions, the fractions TCA precipitated, acid insoluble material trapped on membrane filters, toluene based scintillant added, and the radioactivity assayed in a Packard Tricarb spectrometer. Procedural details are found in METHODS.

Panel A: Nucleolar particle [^{3}H] acid insoluble radioactivity.

Panel B: Nucleolar particle [^{14}C] acid insoluble radioactivity.

B inset: Radioactivity profile (arrowed) redrawn using an expanded scale.

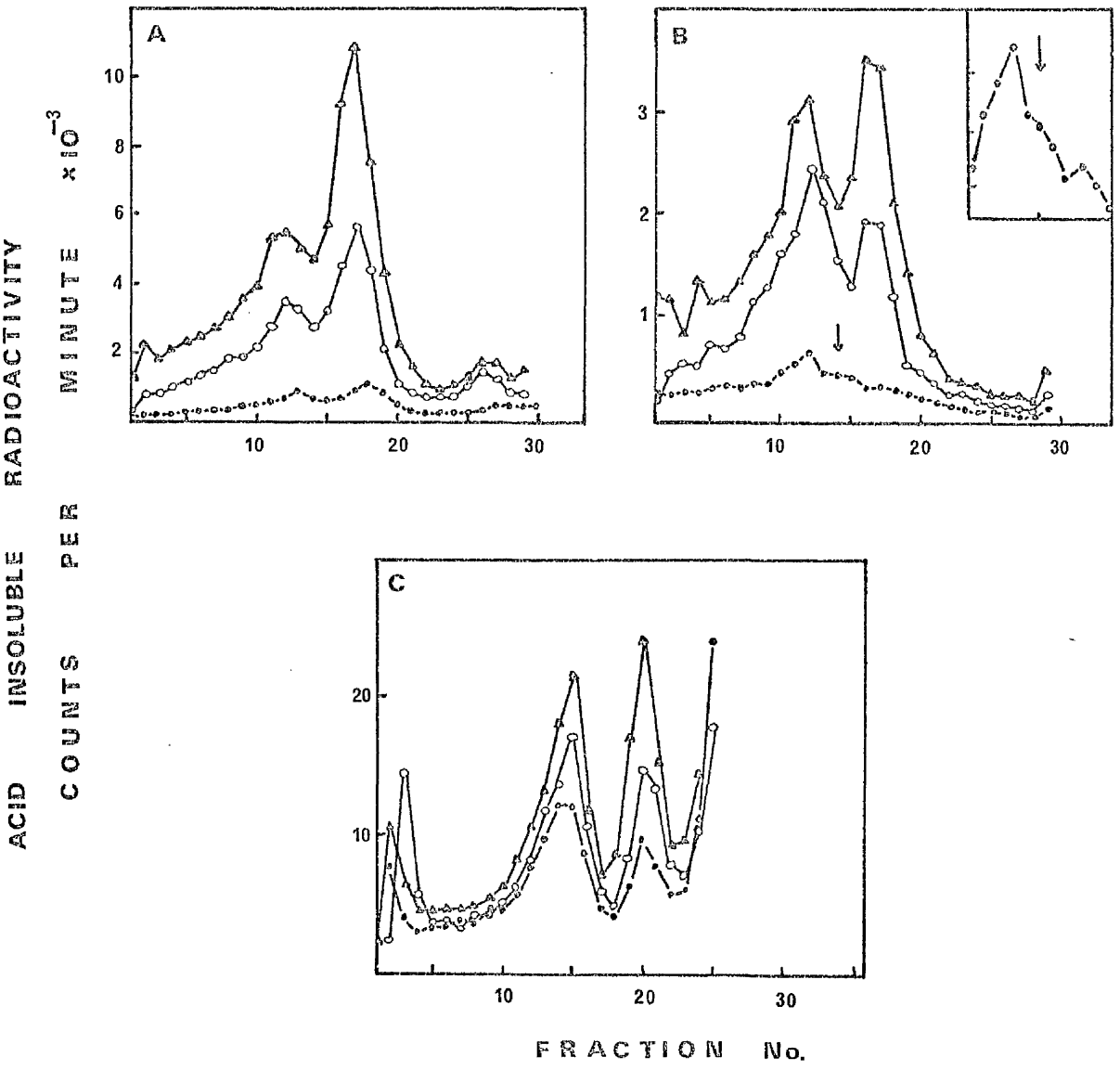
Panel C: Cytoplasmic ribosomal [^{3}H] acid insoluble radioactivity.

Acid insoluble radioactivity in 15 minute sample —●—●—

" " " " 30 " " —○—○—

" " " " 45 " " —▲—▲—

Sedimentation is from right to left.


A
B
C
ACID INSOLUBLE RADIOACTIVITY
COUNTS PER MINUTE x10⁻³
FRACTION No.
10
20
30
2
4
6
8
1
3

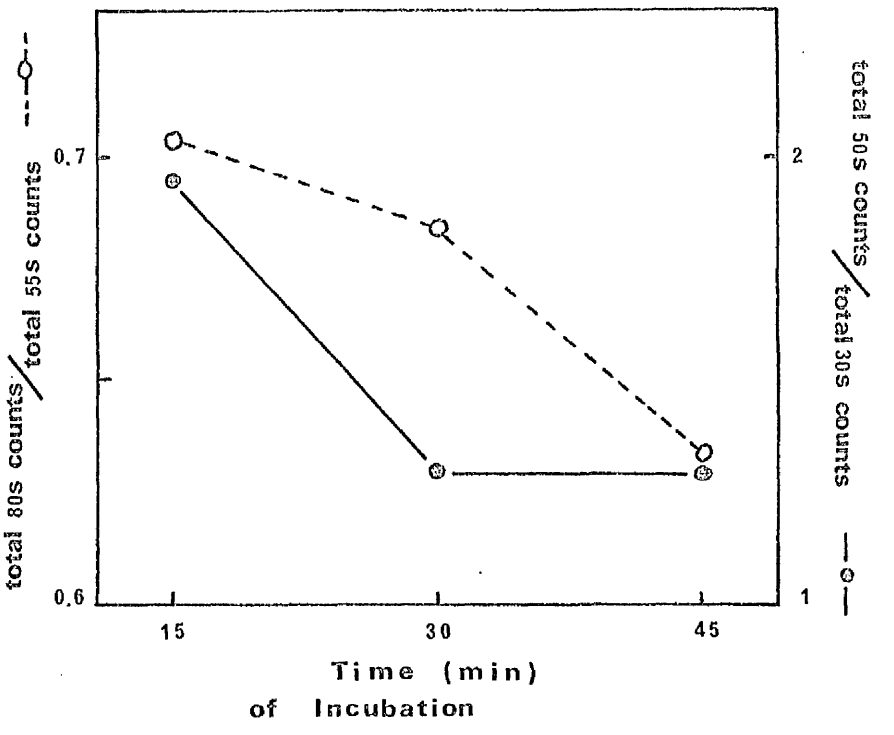


Fig. III (21) Ratio of [^{3}H]leucine radioactivity appearing in 80s/55s nucleolar particles and 50s/30s cytoplasmic ribosomal subunits with time.

The [^{3}H] acid insoluble radioactivity present in the 80s, 55s, 50s and 30s peaks of Fig. III (20) A and C was computed and the ratios of radioactivity appearing in 80s/55s and 50s/30s particles after 15, 30 and 45 minutes incubation determined.

Radioactivity in 80s/55s particles -----O-----

" " 50s/30s " —— ● ——

(Fig. III (21)), there seems also to exist a mechanism of protein addition to, and/or exchange with both 80s and 55s particles in the nucleolus. The possible significance of this finding will be discussed later.

(iii) Cytoplasmic ribosomal protein labelling

After 15 minutes' labelling, [^{3}H]leucine radioactivity in the large subunit is highest with respect to the small subunit, and falls rapidly to reach equilibrium by 30 minutes (Fig. III (21)). This finding suggests that "exchangeable" cytoplasmic proteins (Expt. III B (6)) appear and equilibrate rapidly (within 15 minutes of labelling) on the large subunit. The decline of the 50s/30s [^{3}H]leucine ratio, noted at 30 minutes after the "pulse", probably results from the appearance of newly synthesised small ribosomal subunits from the nucleolus. Complete, newly synthesised large subunits do not appear until at least 60 minutes after commencement of labelling (see Fig. III (16); Vaughan et al., 1967; Vesco and Penman, 1968).

To summarise, this experiment indicates that the optimal incubation time to obtain enrichment of 80s radioactivity in nucleolar particles is 15 minutes or less. However, by this time, only a small percentage of label has been incorporated into nucleolar particles (12.5% of the radioactivity incorporated by 45 minutes), and so the method was discarded as being unlikely to give sufficient label in 80s particles to permit two dimensional fingerprinting.

(2) Effect of hypertonicity on nucleolar particle maturation

By increasing the sodium chloride concentration in cell culture media by a factor of 1.5-2 times, processing of nucleolar 45s RNA is inhibited, although its transcription and methylation are unaffected (Pederson and Robbins, 1970). The possibility of selective enrichment for 80s particles by incubation of cells in high salt concentrations was therefore considered. However, as Fig. III (22) demonstrates, protein synthesis in cells grown in hypertonic (1.5-2 times normal NaCl concentration) medium is inhibited by 90-100%.

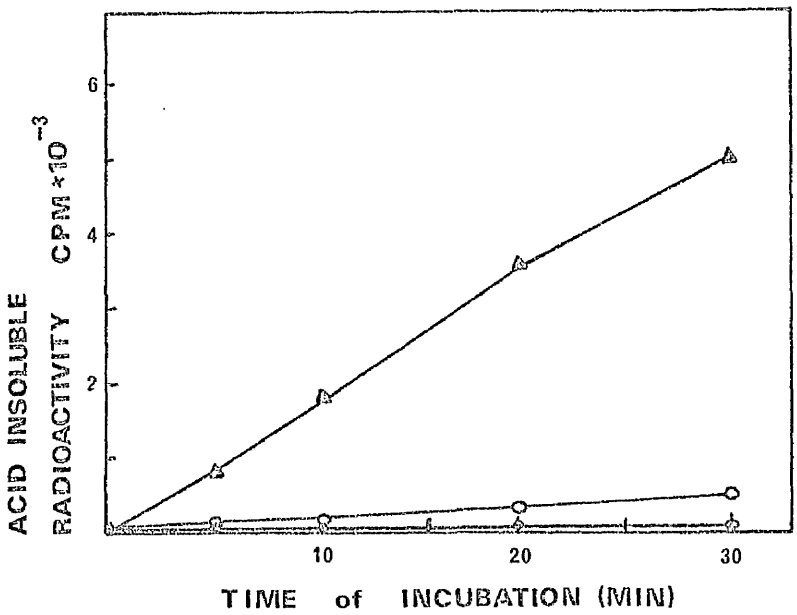


Fig. III (22) Effect of hypertonic medium on protein synthesis in HeLa cells

3 cultures (A, B and C) of 5 x 10^7 HeLa S3 cells, each in 33ml leucine-free Joklik modified EC 10 medium were incubated at 37°C as follows:-

A. Control culture. Isotonic medium (0.116M NaCl).

B. NaCl added to 1.5 x normal concentration

C. " " " 2.0 x " "

To each, after 10 minutes preincubation, were added 10μCi L-[^{14}C] leucine (final specific activity = 312mCi/mmol). 0.2ml aliquots were sampled from each culture at 0, 5, 10, 20 and 30 minutes and added to 0.5ml of 0.1N NaOH to inhibit protein synthesis. The sample was then made 10% with TCA, acid insoluble material collected on membrane filters and radioactivity determined in a Nuclear Chicago low background gas flow counter..

Control culture —— △ —— △ ——

1.5 x isotonic culture —— o —— o ——

2.0 x isotonic culture —— ◎ —— ◎ ——

The effect of increased salt concentration on nucleolar particle labelling in HeLa cells is shown in Fig. III (23). The cells were preincubated in the presence of [^{14}C]leucine to allow uptake of label before increasing the NaCl concentration. The results demonstrate an increase of the 80s/55s radioactivity ratio of 23-28% in the presence of 1.5 x normal NaCl concentration. However, a fall in the absolute uptake of radioactivity of approximately 30% renders this method of 80s particle enrichment ineffective.

The 80s/55s enrichment effect was also noted by Pederson and Kumar (1971) (41% increase of 80s/55s ratio after 45 minutes incubation in hypertonic medium).

(3) Valine starvation and 80s nucleolar particle enrichment

Valine starvation of HeLa cells is known to decrease the rate of processing of nucleolar 45s to 32s RNA (Maden et al., 1969) and to increase the ratio of newly formed small/large ribosomal subunits in the cytoplasm (Maden, 1969). The latter effect could reflect a selective inhibition of synthesis of one or more 50s proteins. On the assumption that ribosomal proteins of the small subunit are first found on the 80s nucleolar particle, valine starvation may be expected to enrich this particle for 30s proteins and increase the ratio of protein labelling on the 80s/55s particles.

Table III (4) shows the result of an experiment performed to test this hypothesis. After one hour and three hours of valine starvation, the ratio of radioactivity incorporated into 80s/55s protein is increased by 7.8% and 15.1% respectively. However, over increasing periods of starvation, incorporation of radioactive amino acids into acid precipitable material declines (Table III (4)),until a basal level of approximately 30% of the normal rate of protein synthesis is reached and maintained by protein turnover (Eagle et al., 1959). Therefore, cells should be pre-starved for valine for the minimum time required to produce the necessary 80s/55s imbalance prior to addition of label.

Following on from the encouraging preliminary experiment described above,

Fig. III (23) Effect of hypertonic medium on 80s/55s nucleolar particle ratio in HeLa cells

Three HeLa S3 cultures (A, B and C), each of 7×10^7 cells in 33ml leucine-free Joklik modified EC 10 were incubated for 15 minutes at 37°C in the presence of 12.5μCi L-[^{14}C]leucine (final specific activity = 312mCi/mmol), and then treated as follows:-

A: no addition. Incubated for further 15 minutes.

B: NaCl added to 1.5 x isotonicity (i.e. to 0.174M). Incubated for further 15 minutes.

C: NaCl added to 1.5 x isotonicity. Incubated for further 30 minutes.

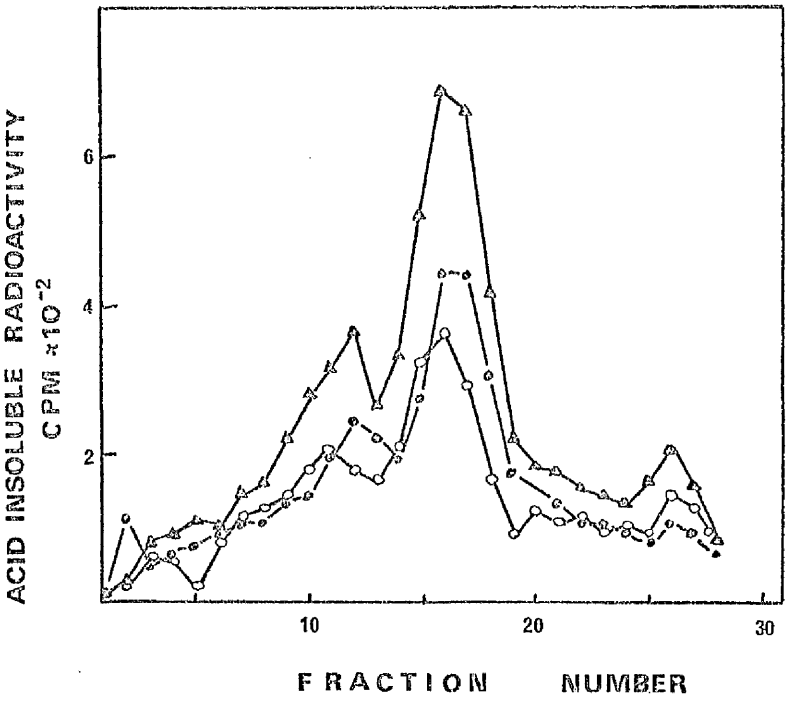
At the end of the specified incubation period, the cultures were chilled and harvested, nucleolar particles were prepared according to METHODS and separated on 36ml 15-30% sucrose gradients in NEB (16 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Each gradient was collected into approximately 30 fractions, the fractions made 10% with TCA, acid insoluble material collected on membrane filters, and assayed for radioactivity in a Nuclear Chicago low background gas flow counter. Technical details are provided in METHODS.

Gradient A —— Δ —— Δ ——

Gradient B —— ◎ —— ◎ ——

Gradient C —— O —— O ——

Sedimentation is from right to left.



	Ratio 80s/55s radioactivity
A. Control	0.416
B. 15' preincubation, 15' hypertonic medium	0.548
C. 15' preincubation, 30' hypertonic medium	0.540

	Total radioactivity (cpm) in 80s particle	Total radioactivity (cpm) in 55s particle	Ratio 80s/55s	% increase of ratio over control
Control (A)	533	1488	0.358	0
1 hour starved (B)	2043	5294	0.386	7.8
3 hours starved (C)	1326	3218	0.412	15.1

Table III (4). Valine starvation and HeLa cell 80s nucleolar particle enrichment.

3 cultures, each containing 10^8 HeLa monolayer cells were incubated as follows:-

Culture A: incubated 45 minutes in 50ml EC 10 containing 10μCi L-[^{14}C] valine (final specific activity = 0.5mCi/mmol)

Culture B: preincubated 1 hour in 50ml valine deficient EC 10 then labelled for 45 minutes with 10μCi L-[^{14}C]valine (final specific activity = 260mCi/mmol) in the same medium.

Culture C: preincubated 3 hours in 50ml valine deficient EC 10 then labelled for 45 minutes with 10μCi L-[^{14}C]valine (final specific activity = 260mCi/mmol) in the same medium.

All cultures were harvested by scraping and nucleolar particles prepared and separated in 36ml 15-30% sucrose gradients (17 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor) as described in METHODS. Each gradient was collected into approximately 30 fractions, the fractions made 10% with TCA, acid insoluble material collected on membrane filters, and radioactivity determined in a Nuclear Chicago gas flow counter. Radioactivity in 80s and 55s peaks was calculated, and the ratio 80s/55s radioactivity determined.

HeLa cells were labelled with [^{35}S]methionine after a period of valine starvation (see legend of Fig. III (24) for experimental details) in an attempt to obtain specific enrichment of 30s proteins on the 80s particle, which was postulated to occur concomitantly with the cytoplasmic enrichment of 30s ribosomal subunits noted by Maden (1969). Nucleolar particles were isolated on sucrose gradients (see profile Fig. III (24) A), and their proteins treated as in METHODS to give the 80s and 55s peptide fingerprints shown (Fig. III (24) B and C). The 55s particle fingerprint resembles that described in Fig. III (14) and demonstrates no abnormality as a result of valine starvation. The 80s fingerprint is also similar to the pattern of 80s peptides in Fig. III (14), and although it contains spots characteristic of some 30s proteins (S3, S9, S14 (faint), S20, S37), specific enrichment for small subunit proteins has not occurred. The significance of the presence of some additional spots (to the left of S3 and above S23 and S28) is not clear.

(4) Glutaraldehyde fixation of nucleolar particles

Formaldehyde (Spirin et al., 1965) and glutaraldehyde (Baltimore and Huang, 1968) cross link, covalently,macromolecules which are in close association, forming a structure which is then stable to exposure to the high salt concentrations of CsCl gradients. Glutaraldehyde has already been used to stabilise nucleolar particles during centrifugation in sucrose gradients (Expt. III A (4)). The legend of Fig. III (25) describes an experiment performed to determine the effectiveness of glutaraldehyde in preventing possible dissociation of 30s proteins from the 80s particle during centrifugation. Table III (5) indicates that 80s particles untreated with glutaraldehyde lose both RNA and protein on sedimentation through sucrose gradients. Glutaraldehyde fixation prevents this loss and produces a 16% increase in the ^{80s}/55s protein radioactivity ratio. However, particles fixed in this way and processed as in METHODS do not provide technically satisfactory two dimensional fingerprints. Most

Fig. III (24) Fingerprints of HeLa cell nucleolar particles labelled in valine deficient medium

2×10^8 HeLa S3 cells were preincubated for 6 hours at 37°C in 300ml valine and methionine-free Joklik modified EC 10, and then labelled for 50 minutes by addition of 1mCi L-[^{35}S] methionine (final specific activity 17Ci/mmol). Nucleolar particles were isolated and separated on a 36ml 15-30% sucrose gradient in NEB (16 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Peak fractions were pooled, proteins extracted and fingerprinted as in METHODS Section (4) C.

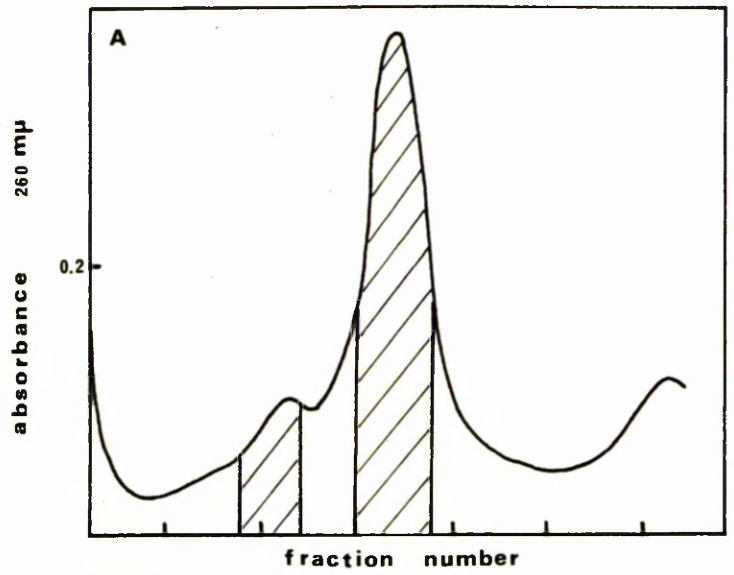
Radioactive peptides were located by autoradiography.

Technical details are provided in METHODS.

Panel A: Absorbance profiles of nucleolar particles showing peak fractions taken. Sedimentation is from right to left.

Panel B: Two dimensional tryptic peptide fingerprint of 80s proteins labelled in valine deficient medium.

Panel C: Two dimensional tryptic peptide fingerprint of 55s proteins labelled in valine deficient medium.


A
absorbance 260 mμ
0.2
fraction number

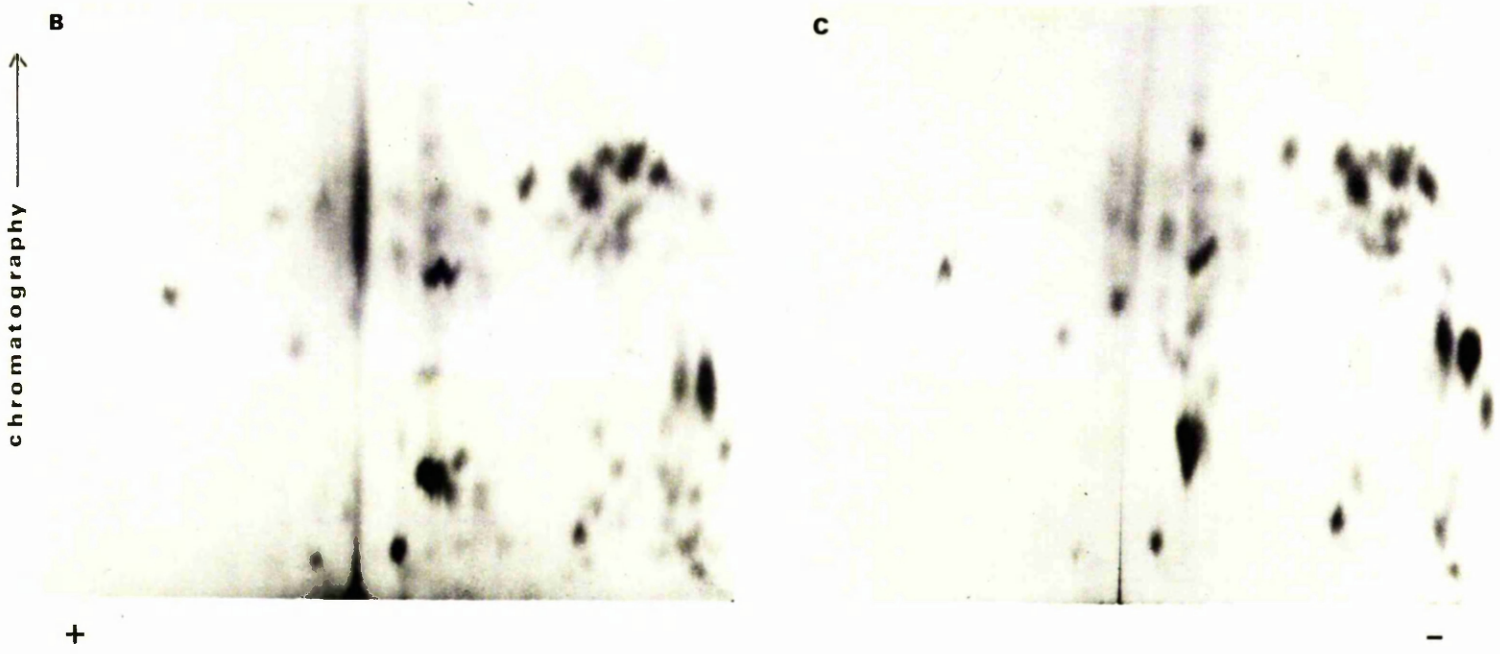

B
C
chromatography
+
−

Fig. III (25) Stabilisation of nucleolar particles by glutaraldehyde

Approximately 6 x 10^7 HeLa monolayer cells were incubated for 1.5 hours at 37°C in 600ml lysine-free EC 10 containing 1μCi [^{14}C]uridine (2.5mCi/mmol) and 250μCi L-[^{3}H]lysine (final specific activity = 250mCi/mmol).
The cells were harvested and nucleolar particles prepared according to METHODS. The extracted particles were divided into two equal aliquots, one aliquot made 8% with glutaraldehyde (Baltimore and Huang, 1968) and both sedimented separately through 36ml 15-30% sucrose gradients in NEB (16 hours, 70,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The gradients, after centrifugation, were collected into approximately 30 fractions, each fraction made 10% with TCA, acid insoluble material collected on membrane filters and radioactivity determined in a Packard Tricarb spectrometer using toluene based scintillant. All technical details are provided in METHODS.

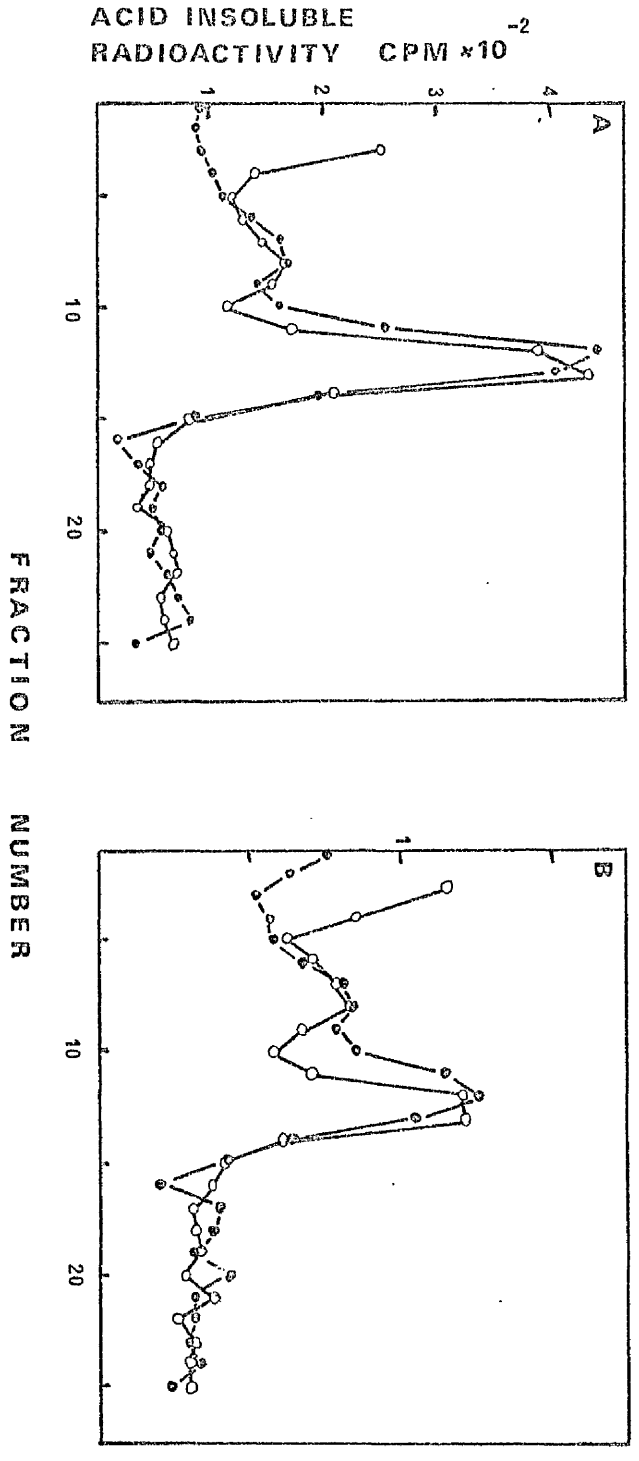
Panel A: acid insoluble [^{3}H] radioactivity

Panel B: acid insoluble [^{14}C] radioactivity

Untreated nucleolar particles —●—●—

Glutaraldehyde fixed nucleolar particles —○—○—

Sedimentation is from right to left.


ACID INSOLUBLE
RADIOACTIVITY CPM ×10 -2
A
B
FRACTION NUMBER

	Total protein radioactivity in particle (cpm)	80s / 55s ratio	Total RNA radioactivity in particle (cpm)	80s / 55s ratio
80s + glutaraldehyde	825		420	
		0.616		0.927
55s + glutaraldehyde	1338		453	
80s no glutaraldehyde	830		370	
		0.530		0.667
55s no glutaraldehyde	1565		555	

Table III (5) Stabilisation of nucleolar particles by glutaraldehyde

The experiment was performed as described in Fig. III (25). [^{3}H]lysine and [^{14}C]uridine radioactivity in glutaraldehyde fixed and unfixed 80s and 55s nucleolar particles was calculated and from these data, the ratio of [^{3}H] or [^{14}C] radioactivity in 80s/55s particles was derived.

radioactive material does not migrate either on electrophoresis or chromatography, and it has been concluded that the covalent linkage of RNA with protein which is produced by glutaraldehyde, results in a complex which is resistant to the action of ribonuclease and/or trypsin, and cannot be fractionated by the two dimensional fingerprinting technique.

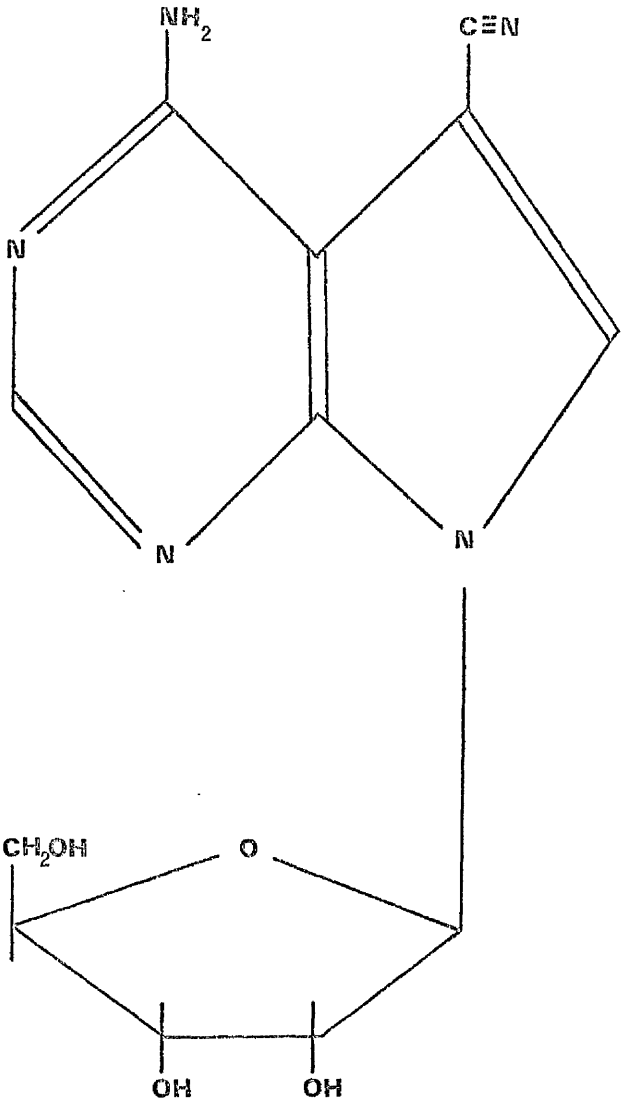
(5) Effect of toyocamycin on nucleolar RNA and nucleolar particle maturation

Low concentrations of toyocamycin, an adenosine analogue which is incorporated into RNA (Fig. III (26)),are known to inhibit mammalian cell rpreRNA maturation. This results in accumulation of normally methylated 45s RNA in the nucleolus (Tavitian et al., 1968, 1969; Sverak et al., 1970). Amino acid incorporation into acid precipitable material is not inhibited by short exposures of HeLa cells to the drug (Tavitian et al., 1968; Fig. III (27)). At 0.12µg/ml culture medium, conversion of 45s to 32s nucleolar RNA is curtailed, and at 1.0µg/ml, 45s RNA synthesis is strongly depressed (Fig. III (28)). The experiment described in the legend of Fig. III (29) was designed to utilise the inhibitory action of toyocamycin on 45s RNA to prepare 80s nucleolar particles in high yield, and uncontaminated by 55s material. The results show that in the presence of toyocamycin, new nucleolar particle formation is inhibited although, at the concentration of toyocamycin used (0.2µg/ml), protein synthesis and 45s RNA production are continuing. The failure of RNA and protein to complex together may result from defective toyocamycin-containing 45s RNA tertiary structure development. Alternatively it may follow another as yet undefined effect of toyocamycin on the cell, by which the RNA and proteins synthesised do not gain proximity to each other to enable particle formation. We favour the former view.

SECTION III D. SUMMARY

A number of procedures have been used in an attempt to produce selective enrichment, either of 80s with respect to 55s nucleolar particles, or of 30s with respect to 50s proteins on the 80s precursors (or both).

(1) The ratio of ^{80s}/55s radioactivity is higher after a short (15 minute)



TOYOCAMYCIN

4 – amino – 5 – cyano – 7 – ß – D – ribofuranosy – pyrrolo (2,3 – d) pyrimidine

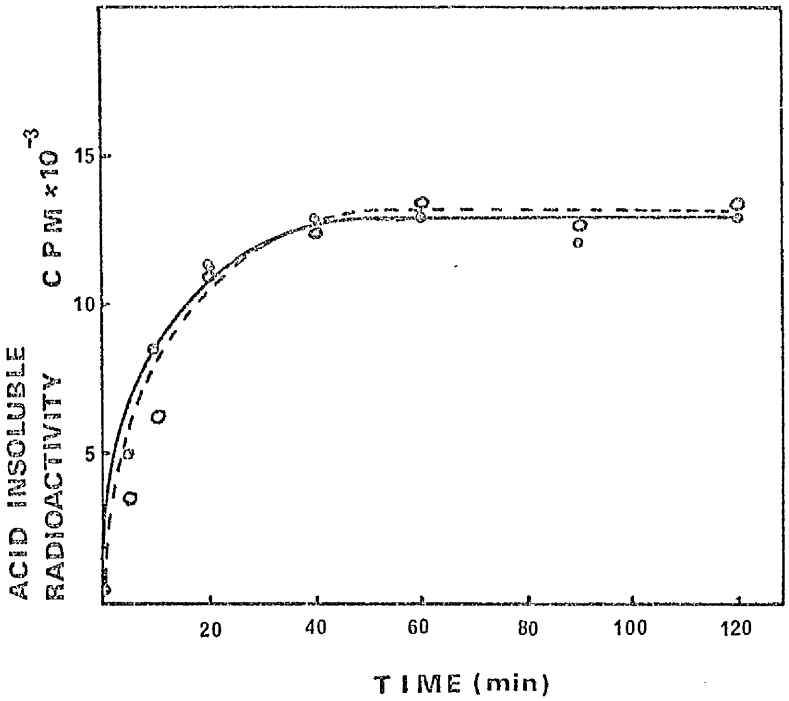


Fig. III (27) Effect of toyocamycin on HeLa cell protein synthesis

Two aliquots of 5 x 10^7 HeLa S3 cells were suspended in 50ml of leucine-free Joklik modified EC 10. To the test culture was added toyocamycin to 1.0µg/ml and both cultures were preincubated for 30 minutes before the addition to each of 12.5µCi L-[^{14}C] leucine (312mCi/mmol). 0.2ml aliquots were sampled from each culture at times 0, 5, 10, 20, 40, 60, 90 and 120 minutes, added to 0.5ml of 0.1N NaOH, made 10% with TCA and acid insoluble material collected on membrane filters. Radioactivity was measured on a Nuclear Chicago low background gas flow counter.

Control culture —— ⊙ —— ⊙ ——

Culture containing toyocamycin (1µg/ml) ---- o ---- o ----

Fig. III (28) Effect of toyocamycin on HeLa cell nucleolar RNA synthesis and maturation

5 rotating 80oz. winchester bottles, each containing 10^8 HeLa monolayer cells in 200ml EC 10 were incubated at 37°C for 30 minutes after addition of toyocamycin as follows:-

1. Control culture. No toyocamycin added.

2. 0.125µg toyocamycin/ml.

3. 0.250µg toyocamycin/ml.

4. 0.500µg toyocamycin/ml.

5. 1.0µg toyocamycin/ml.

To each was then added 5µCi [^{3}H]uridine (5Ci/mmol), and incubation continued for 60 minutes. The cells were harvested, nucleolar RNA extracted as in METHODS and separated on 36ml 15-30% sucrose gradients in LETS (16 hours, 62,000g, +20°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Each gradient was collected into approximately 30 fractions, the fractions made 10% with TCA, acid insoluble material collected on membrane filters and radioactivity determined in a Packard Tricarb spectrometer using toluene based scintillant. Technical details are provided in METHODS.

Panel A: Culture 1. Control cells.

Panel B: Culture 2. 0.125µg toyocamycin/ml.

Panel C: Culture 3. 0.250µg toyocamycin/ml.

Panel D: Culture 4. 0.500µg toyocamycin/ml.

Panel E: Culture 5. 1.0µg toyocamycin/ml.

Sedimentation is from right to left.

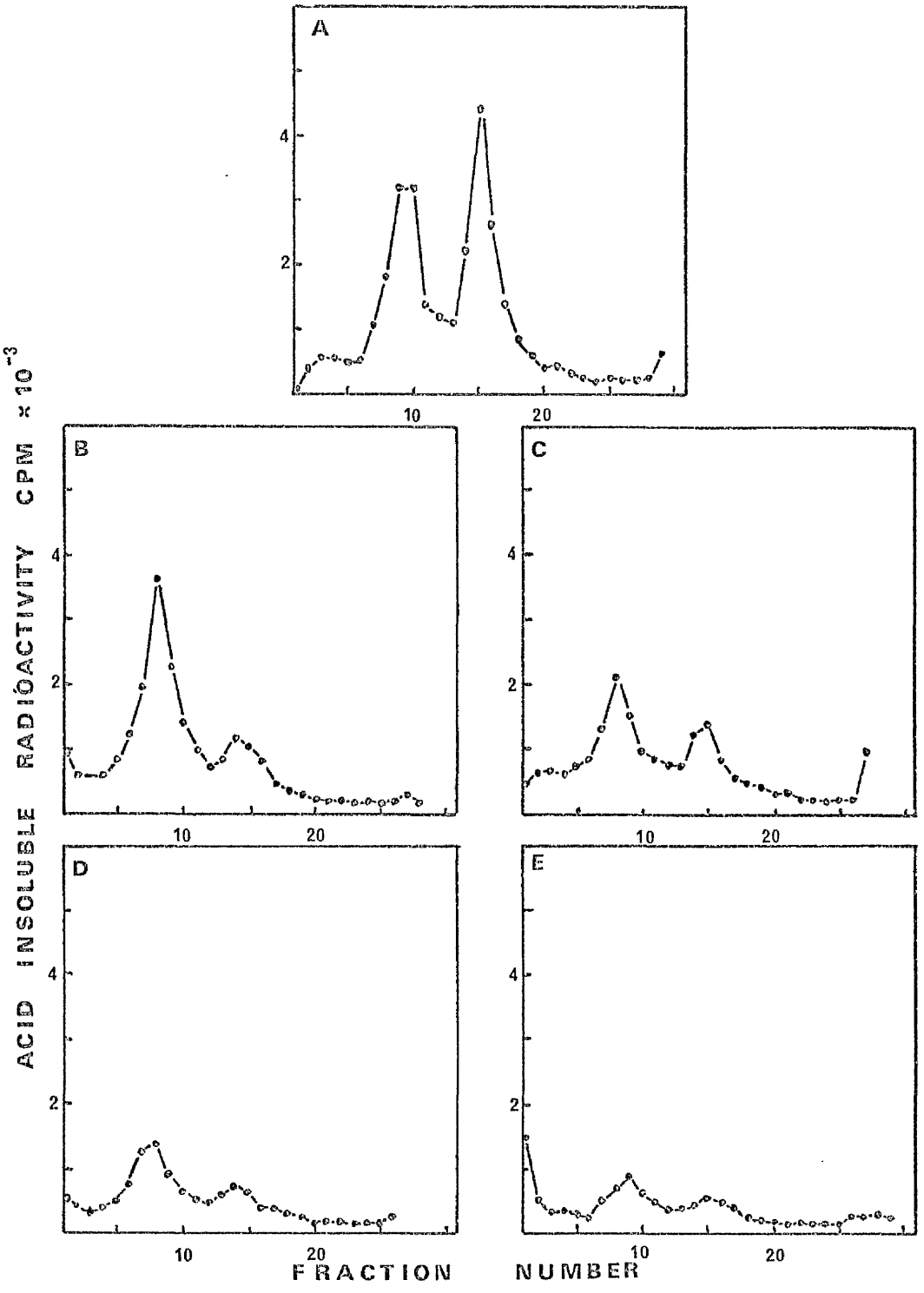

A
B
C
D
E
4
2
10
20
ACID INSOLUBLE RADIOACTIVITY CPM x 10⁻³
FRACTION NUMBER

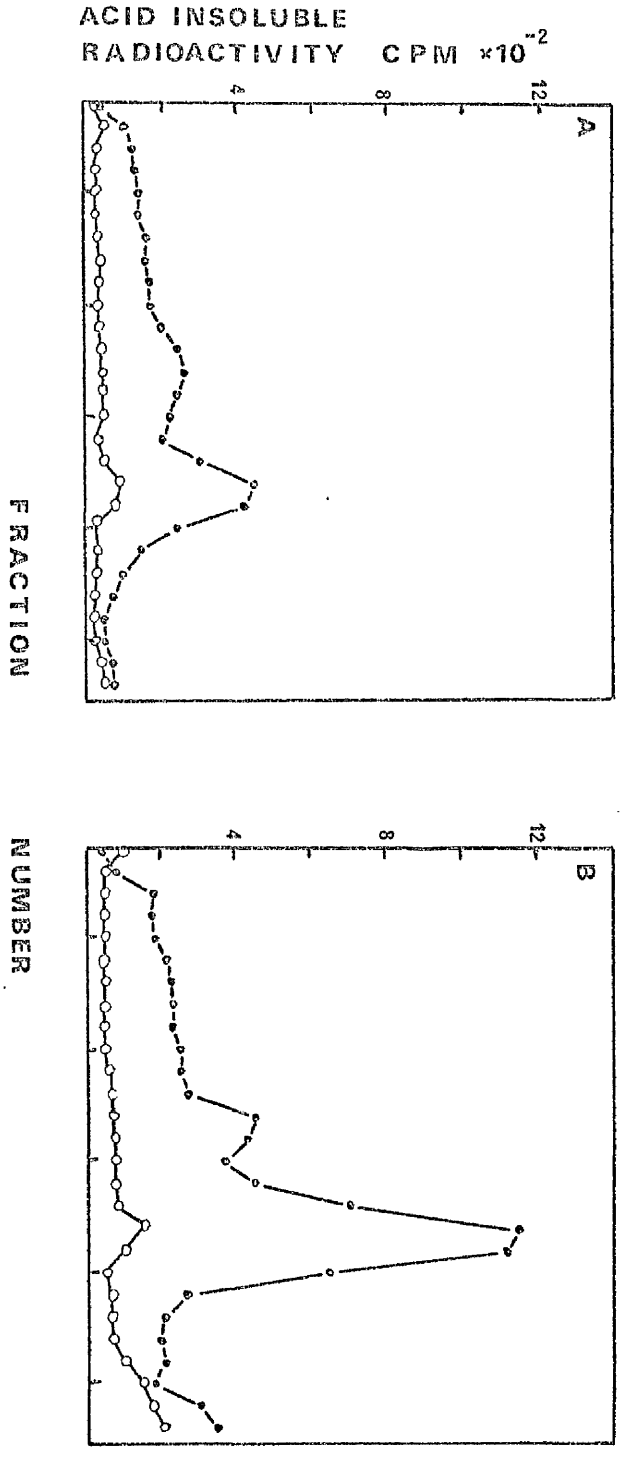
Fig. III (29) Effect of toyocamycin on HeLa cell nucleolar particle synthesis

4 cultures (A, B, C and D) each of 5 x 10^7 HeLa cells were grown at 37°C in 80oz. winchester bottles containing 200ml EC 10. To C and D were added 0.125µg toyocamycin/ml and preincubation of all four cultures continued for 30 minutes. The medium was then changed to 50ml leucine-free EC 10 containing 20µCi L- [^{3}H] leucine (5Ci/mmol) per bottle, and again 0.125µg toyocamycin/ml were added to C and D. A and C were harvested after 30 minutes further incubation, and B and D after 120 minutes. Nucleolar particles were isolated from all cultures and separated on 36ml 15-30% sucrose gradients in NEB (16 hours, 70,000g, + 4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). The gradients were collected into approximately 30 fractions, the fractions made 10% with TCA, and acid insoluble material collected on membrane filters. Radioactivity in each fraction was measured in a Packard Tricarb spectrometer using toluene-based scintillant. Technical details are found in METHODS.

Panel A: Radioactive profiles of control —o—o— and toyocamycin treated —o—o— nucleolar particles extracted after 30 minutes [^{3}H]leucine labelling.

Panel B: Radioactive profiles of control —o—o— and toyocamycin treated —o—o— nucleolar particles extracted after 120 minutes [^{3}H]leucine labelling.

Sedimentation is from right to left.


ACID INSOLUBLE
RADIOACTIVITY CPM ×10⁻²
4
8
12
A
B
FRACTION NUMBER

"pulse" with [^{3}H]leucine than after longer (30-45 minute) "pulses". However, the low yield of radioactive material obtained after a 15 minute "pulse" precludes the use of this method to obtain 80s particle enrichment prior to two dimensional peptide fingerprinting.

(2) Growth of cells in hypertonic medium produces valuable selective enrichment for 80s nucleolar particles by inhibiting nucleolar processing of 45s rpreRNA. Unfortunately, hypertonic medium inhibits protein synthesis in cultured cells and so this procedure does not provide a good yield of high specific activity nucleolar 80s proteins.

(3) Valine starvation induces enrichment of 80s over 55s nucleolar material extracted from HeLa cells. This is not the result of accumulation of 30s proteins on the enriched particle.

(4) Glutaraldehyde fixation inhibits 80s nucleolar particle degradation (see Expt. III A. (4)), and increases the ratio of protein radioactivity in 80s/55s particles by 16%. However, the stabilised particles are resistant to RNase and tryptic digestion, and their proteins cannot be fingerprinted by the procedure described in METHODS.

(5) Toyocamycin in low concentrations causes selective nucleolar enrichment of 45s rpreRNA without inhibiting cell protein synthesis. However, the modified rpreRNA synthesised in the presence of toyocamycin does not associate with ribosomal proteins and therefore during toyocamycin treatment, 80s and 55s nucleolar particles effectively disappear from the cells' nucleoli.

None of the above modifications to cell growth conditions or labelling procedures has resulted in a useful degree of enrichment of 80s over 55s nucleolar particles. This failure has limited, to some extent, the conviction with which certain earlier data on 80s nucleolar particle structure can be interpreted.

SECTION III E - Fingerprinting studies of ribosomal subunit proteins derived from various species.

Comparative studies of ribosomal subunit proteins have met with limited success because of the restricted resolving power of current fractionation procedures. However, published results suggest that ribosomes from related species contain basically similar proteins, although some minor interspecies differences may be found (Waller, 1964; Mutolo et al., 1967; Di Girolamo and Cammarano, 1968). The improved resolution of the two dimensional fingerprinting technique was used to compare ribosomal subunit proteins of hamster (BHK-21/C13), human (HeLa), and mouse (L929) cell lines, of chick embryo primary cultures, and of yeast (I am indebted to Dr. J.R. Warner for providing yeast ribosomal proteins).

Figs. III (30) and (31) show the results of such studies. It can be seen that a strong similarity exists between patterns of respective subunit proteins of ribosomes derived from mammalian sources (HeLa - Fig. III (9); BHK and L929 - Fig. III (30)). The small subunit patterns from these organisms are basically the same, while those obtained from the large subunit proteins of HeLa and BHK-21/C13 are also very similar. The L929 large subunit differs slightly from the 50s HeLa and BHK-21/C13 patterns in the region of spots L30 (two extra spots in L929) and L21-24 (4 extra spots in L929), but this difference reflects only a minor modification in the L929 large subunit protein complement. Incidentally, fingerprints of 28s rRNA from L929 cells show minor methylation modifications when compared to the corresponding pattern in HeLa or BHK-21/C13 cells (M. Salim, unpublished). It is tempting to suggest that these modifications of the primary structures of RNA and protein from the same ribosomal subunit indicate an alteration of quaternary structure and of RNA-protein interaction on that subunit.

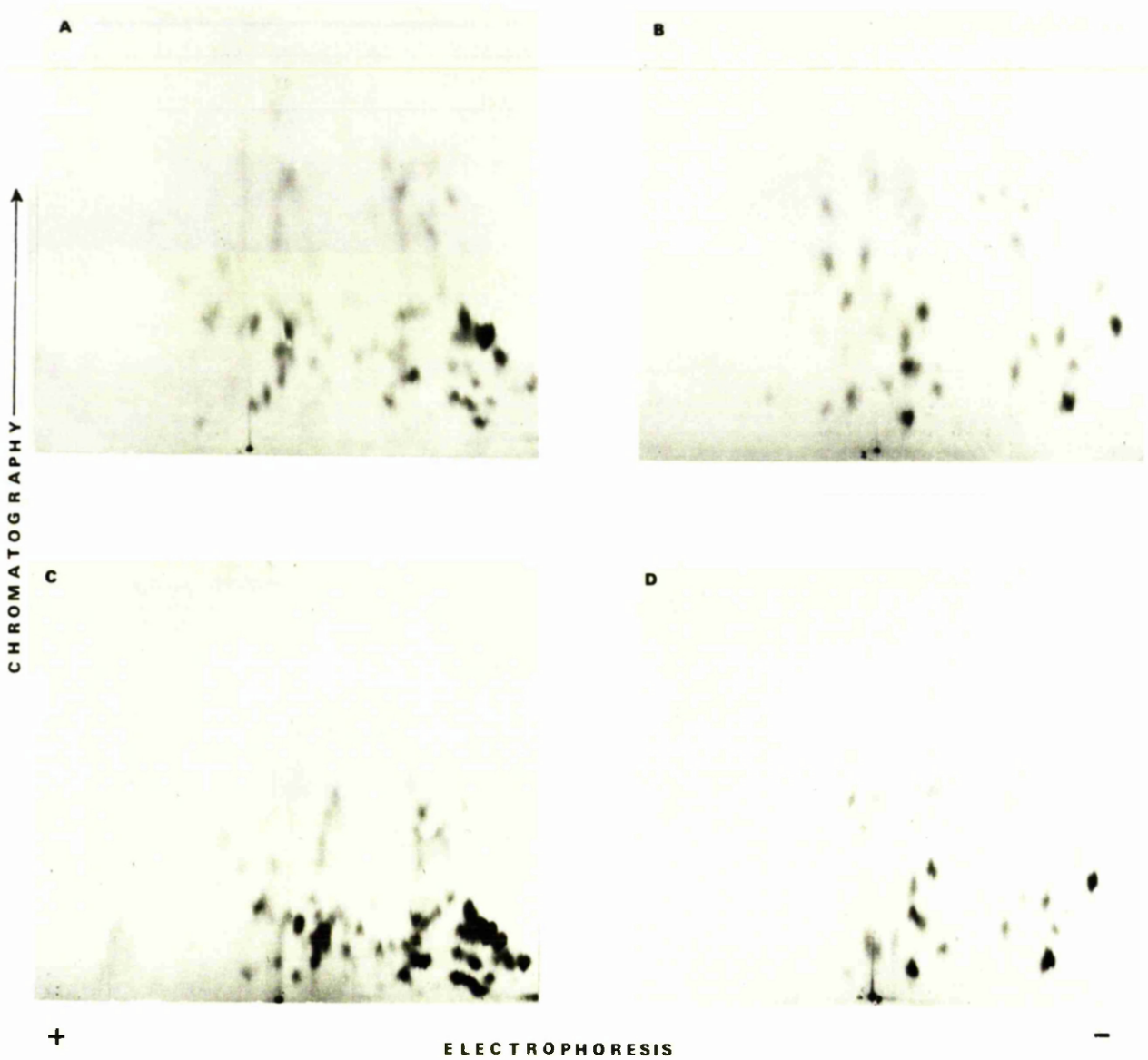

A
B
C
D
CHROMATOGRAPHY
+
ELECTROPHORESIS
−

Fig. III (30) Two dimensional peptide fingerprints of ribosomal subunit proteins from BHK-21/C13 and L929 cells

Monolayer cultures of BHK-21/C13 and L929 cells, seeded (25×10^6 cells) in 80oz. winchester bottles containing 200ml EC 10 were grown, after addition of 0.8mCi L-[^{35}S]methionine (final specific activity = 80mCi/mmol), for 72 hours at 37°C. The cells were harvested by trypsinisation, ribosomes isolated from each culture, dissociated by EDTA, and separated into subunits in 36ml 15-30% sucrose gradients in NEB (16 hours, 80,000g, + 4°C in a Beckman L2 ultracentrifuge with SW 27 rotor). Subunit peak fractions were pooled, their proteins extracted, and fingerprinted according to METHODS, Section (4) C. Radioactive peptides were located by autoradiography.

Panel A: Two dimensional tryptic peptide fingerprint of BHK-21/C13 50s subunit proteins.

Panel B: Two dimensional tryptic peptide fingerprint of BHK-21/C13 30s subunit proteins.

Panel C: Two dimensional tryptic peptide fingerprint of L929 50s subunit proteins.

Panel D: Two dimensional tryptic peptide fingerprint of L929 30s subunit proteins.

Fig. III (31) Two dimensional peptide fingerprints of ribosomal subunit proteins from chick and yeast cells

3 monolayer primary cultures, each containing 7×10^7 cells derived from chick embryos as described in METHODS were each labelled with 300μCi L-[^{35}S]methionine (final specific activity = 60mCi/mmol) for 18 hours at 37°C. The cells were harvested by scraping, ribosomes isolated, and dissociated by EDTA. The subunits were separated on a 36ml 15-30% sucrose gradient in NEB (16 hours, 80,000g, +4°C in a Beckman L2 ultracentrifuge with SW 27 rotor), 30s and 50s peak fractions pooled, and their proteins extracted and fingerprinted in two dimensions as in METHODS Section (4) C.

Yeast ribosomal subunit proteins, prepared by the method of Warner (1971) were generously provided by Dr. J.R. Warner. These were also fingerprinted as in METHODS Section (4) C. Radioactive spots were located by autoradiography.

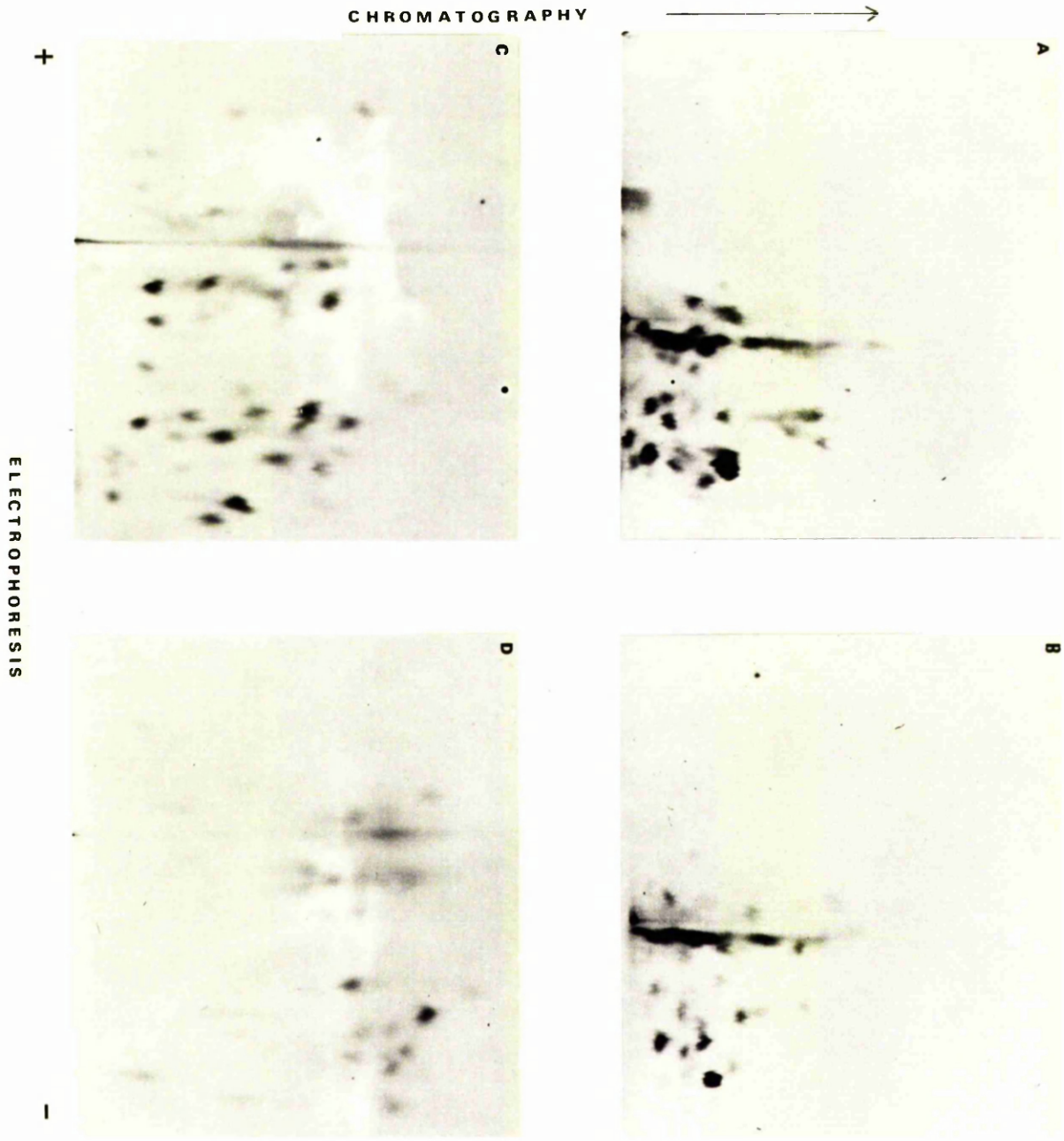
All technical details are provided in METHODS.

Panel A: Two dimensional tryptic peptide fingerprint of chick 50s ribosomal subunit proteins.

Panel B: Two dimensional tryptic peptide fingerprint of chick 30s ribosomal subunit proteins.

Panel C: Two dimensional tryptic peptide fingerprint of yeast 50s ribosomal subunit proteins.

Panel D: Two dimensional tryptic peptide fingerprint of yeast 30s ribosomal subunit proteins.


CHROMATOGRAPHY
ELECTROPHORESIS
+
−
A
B
C
D

With increasing evolutionary divergence, it is apparent that the ribosomal subunit proteins show increasing pattern differences. For example, the chick (avian) subunit fingerprints show considerable variation from the mammalian patterns (cf. Figs. III (31) and (32) A and B); and the yeast peptide "maps" bear no features in common with the animal cell ribosomal protein fingerprints (cf. Figs. III (31) and (32) C and D). These results confirm, using an independent technique, other data derived by polyacrylamide gel electrophoresis and suggest that the degree of similarity of ribosomal proteins from different species is a reflection of the evolutionary relationships which obtain between these species.

DISCUSSION.

SECTION IV - DISCUSSION

IV A. Technical considerations

The two dimensional fingerprinting technique described in this study was used to answer a number of specific questions relative to the structure and formation of mammalian ribosomes. Prior to its application to this problem, the technique was examined in several ways to justify its use in this project. These preliminary studies are evaluated below.

(1) Sensitivity and reproducibility of the fingerprinting technique

Reference has already been made to the fact that at the commencement of this work, the most sensitive protein fractionating technique available for the study of eukaryotic ribosomal proteins was unidimensional polyacrylamide gel electrophoresis (Section I D.). However, this technique was not adequately sensitive for the purposes of this project (Fig. III (8); see also Kedes et al., 1966; Low and Wool, 1967; Di Girolamo and Cammarano, 1968; Clegg and Arnstein, 1970; . King et al., 1971). This is because the migration distance of a protein in a sodium dodecyl sulphate-polyacrylamide gel is inversely proportional to the logarithm of its molecular weight (Weber and Osborn, 1969), and since many ribosomal proteins have coincident molecular weights, their separation on acrylamide gels is incomplete. Conversely, ribosomal peptide fractionation by the two dimensional fingerprinting technique depends on the detailed chemical structure of the subunit proteins. Therefore, although proteins of similar molecular weights will co-migrate on acrylamide gels, they may, because of their different primary structure, provide completely different tryptic fingerprints. In the light of the above knowledge, it was important initally to determine whether the fingerprinting technique was sufficiently sensitive and gave adequately reproducible results to permit meaningful inferences to be drawn from the data which it provided.

Fig. III (9) demonstrates the sensitivity of the fingerprinting method. Spots characteristic of the large and small ribosomal subunit proteins can be differentiated on the respective fingerprints (see Fig. III (10)), and, moreover, it is evident that the general configuration of the fingerprint pattern from each subunit is different (cf. Figs. III (9) A and B). Such a degree of resolution was an improvement over that found by polyacrylamide gel electrophoresis, and since the results were reproducible, it was considered that this method would constitute a useful procedure for the study of eukaryotic ribosome formation.

(2) Definition of a ribosomal protein

No completely adequate definition of a eukaryotic structural ribosomal protein exists (see Nomura, 1970), but since the purpose of this investigation was to study the structural proteins of the HeLa cell ribosome and to gain information about the sequence of their addition to pre-ribosomes in the cell nucleolus, it was important to define clearly what proteins were to be designated "ribosomal proteins". Such proteins were chosen, arbitrarily, to be those which exist on ribosomes purified by magnesium precipitation, and dissociated by EDTA treatment according to the method of Warner (1966). Reference has already been made to the reasons for this choice. First, this simple method of preparation provides reproducibly high yields of essentially uncontaminated 50s and 30s ribosomal subunits (Expt. III A.(2)). Secondly, since the method employs EDTA as dissociation agent, data obtained by its use can be compared directly with similar information derived from EDTA-prepared nucleolar particles (Warner and Soeiro, 1967). The validity of the proposed definition of a ribosomal protein was tested in two ways. Peptide "maps" of the EDTA-dissociated subunit proteins were compared with corresponding fingerprints of proteins isolated from KCl-derived subunits (Fig. III (11)). Since the fingerprints are very similar, it may be deduced that respective subunits prepared by either dissociation process contain proteins with the same primary structures.

Moreover, since salt-washing is used routinely to purify prokaryotic ribosomes (Kurland, 1966; Spirin and Gavrilova, 1969; Kaltschmidt and Wittmann, 1970a; Hindennach et al., 1971), and has been shown to be effective in removing loosely bound proteins from eukaryotic ribosomal subunits (Warner, 1966; Olsnes, 1971) and from purified RNA (Baltimore and Huang, 1970), it seems reasonable to suggest that the salt-washing procedure removes non-ribosomal proteins from the subunits without destroying the particles' ability to synthesise proteins in vitro (Martin and Wool, 1968). Therefore, by extrapolation, EDTA-dissociated ribosomal subunits contain the minimum number of proteins necessary for protein synthesis, and probably few, if any, more. Confirmation of this tentative conclusion was made by the procedure detailed in Expt. III A.(3) which showed that non-specific protein contamination of ribosomes during their isolation by magnesium precipitation is negligible (Fig. III (4)). From these investigations, it was concluded that the two dimensional fingerprints of Fig. III (9) A and B are representative of peptide "maps" of ribosomal subunit proteins, uncontaminated by non-specific peptides.

(3) Possible artefacts induced by peptide fingerprinting

Before employing the two dimensional fingerprinting technique to study the mechanism of eukaryotic ribosome assembly, it was important to ensure, first that the radioactive marker (L- [^{35}S]methionine) used to analyse the proteins was, in fact, found in all or most ribosomal proteins and was not confined to a small, possibly unrepresentative group of polypeptides. This was done by the procedure described in Expt. III A (6) and the results showed a fairly even distribution of [^{35}S]methionine through most proteins of each subunit. Therefore, tryptic fingerprints of [^{35}S]methionine-labelled HeLa ribosomal proteins contain peptide representation from most of these proteins.

Secondly, it was essential to determine that the method of analysis of the isolated subunit proteins did not induce artefacts into the fingerprints obtained. Such artefacts are most likely to derive from the protein

extraction step (METHODS (4) C) in which pancreatic ribonuclease is used to free the ribosomal proteins from rRNA. LiCl/urea-extracted proteins (Leboy et al., 1964) provided essentially similar fingerprints to those derived by RNase treatment of the subparticles (Cf. Figs. III (9) and III (12)). It is evident, therefore, from these data, that [^{35}S] methionine is a suitable probe for use in this study, and that the ribonuclease digestion procedure does not, during ribosomal protein extraction, give rise to artefacts in the two dimensional fingerprints.

(4) Effects of ribosomal subunit cross-contamination on the interpretation of two dimensional peptide fingerprints

An important aim of this project was to utilise the two dimensional peptide fingerprinting technique to identify, in total tryptic hydrolysates of ribosomal subunit proteins, peptides characteristic of each subunit. Subunit-specific peptides could then be sought in nucleolar particles. In essence, such a project requires that, first, the subunit peptide patterns are unequivocally different from each other and, secondly, that cross-contamination of one subunit by the other does not inhibit identification of particular subunit-specific peptides.

Fig. III (9) demonstrates that the characteristic 30s and 50s fingerprints contain an adequate number of subunit-specific peptides (Fig. III (10)), and if there was any cross-contamination, this was so slight as not to be evident from these fingerprints. This finding made unnecessary any further purification of EDTA-derived subunit proteins. Conversely, visible cross-contamination of 60s with 40s subunit peptides (Fig. III (11) A) rendered the KCl dissociation technique unsuitable for the purpose of this investigation.

Finally, an interesting phenomenon is evident from the fingerprints of Fig. III (9) and was reproducible under the standard experimental conditions described in METHODS. Marked differences in labelling intensity can be seen between different peptides in both 50s and 30s fingerprints. Such differences may result from a variety of causes,

including -

(i) incomplete hydrolysis of some potentially susceptible peptide bonds during tryptic digestion.

(ii) the existence of more than one methionine residue in certain peptides.

(iii) the occurrence of some peptides (possibly simple di- or tri-peptides) in more than one ribosomal protein.

(iv) selective partial loss of some protein during ribosomal preparation.

(v) multiple occurrence of some proteins per single ribosome (in the case of strongly labelled peptides).

(vi) heterogeneity among ribosomes with respect to protein content.

Fractionation of the ribosomal proteins to a point where more thorough protein chemistry becomes possible will be required to distinguish between these possibilities. Meanwhile, the fingerprints, reproducible under standard experimental conditions, form a useful basis for comparative experiments.

Fig. III (32) Proposed scheme of the mechanism of HeLa cell ribosome formation

Note:-

1. The number at the top of each "particle" represents the sedimentation coefficient of that particle.

2. 5s RNA becomes associated with the large ribosomal subunit precursor in the nucleolus, but the state at which this association occurs is not yet clear.

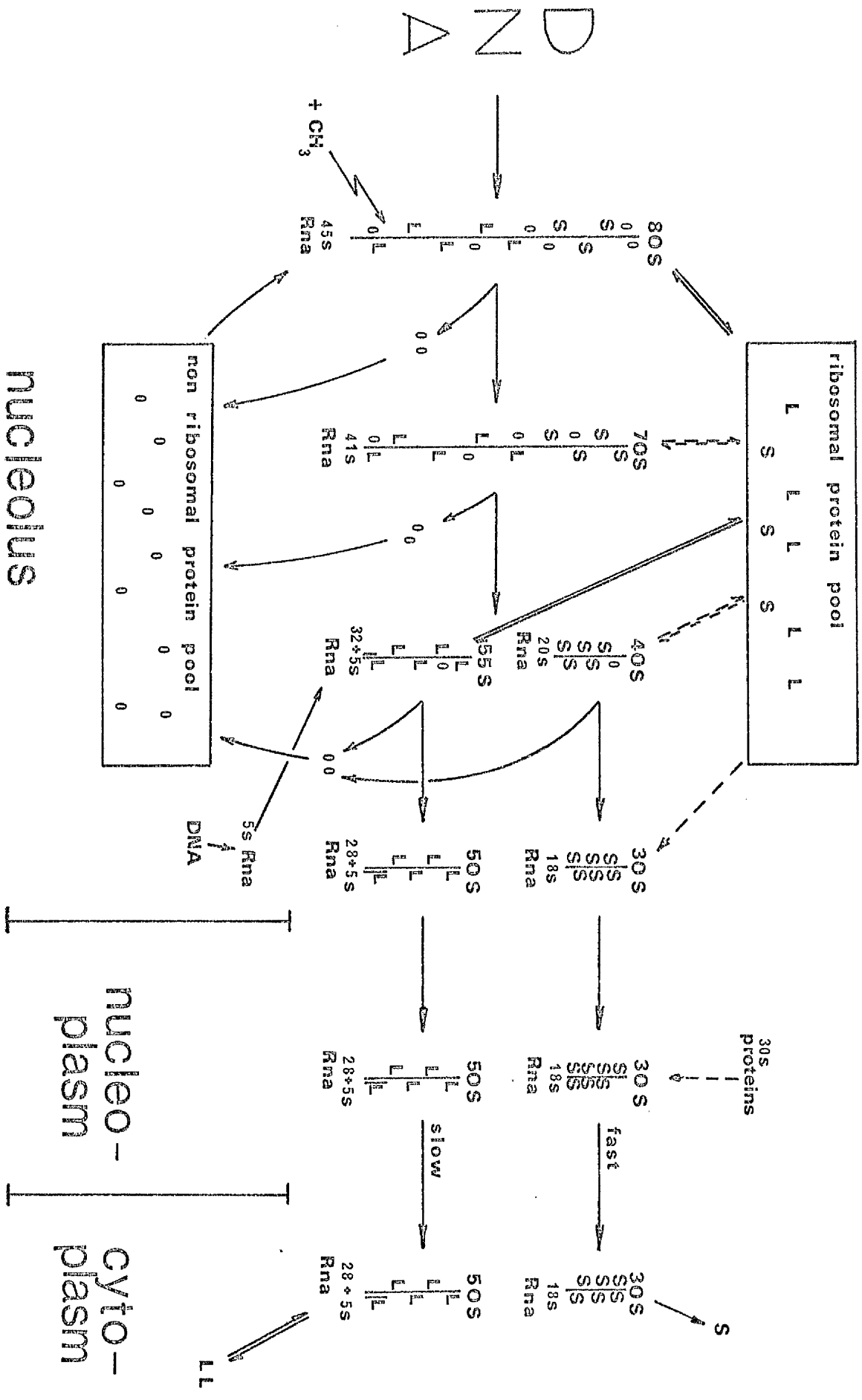
3. Broken lines indicate tentative sites of protein-particle interaction in the nucleolus.

4. L = large subunit ribosomal protein

   S = small " " "

   O = non ribosomal protein associated with nucleolar particles

   + CH_3 indicates a site of methylation


nucleus
nucleo-plasm
cyto-plasm
ribosomal protein pool
non ribosomal protein pool
DNA
5s Rna
+ CH3
45s Rna
80S
70S
41s Rna
55S
32+5s Rna
40S
20s Rna
50S
28+5s Rna
30S
18s Rna
30s proteins
slow
fast
S
LL

IV B. Ribosome formation in HeLa cells

Although eukaryotic ribosomal proteins appear to be synthesised in the cytoplasm (Heady and McConkey, 1970; Craig and Perry, 1971), the work of Warner and Soeiro (1967) indicates that most 50s ribosomal proteins, at least, combine with rpreRNA in the nucleolus. However, correspondence between acrylamide gel electrophoresis patterns of total nucleolar and ribosomal proteins is not exact (Shepard and Noland, 1968; Mundell, 1968) and attempts to demonstrate, by acrylamide gel electrophoresis, 30s ribosomal subunit proteins in the total nucleolar protein "pool" have proved unsuccessful (Maden and Warner, unpublished). Nevertheless, the fact that 18s RNA apparently passes through the nucleoplasm and enters the cytoplasm in the form of ribonucleoprotein complexes (Girard et al., 1965) which are stable in the presence of high salt concentrations (Vaughan et al., 1967) suggests that, like 50s ribosomal subunit formation, 30s particle assembly commences in the nucleolus. The two dimensional fingerprinting system developed during this project was used to test this prediction, and also to examine other aspects of ribosome structure and assembly. The results obtained in the study have been summarised in diagrammatic form (Fig. III (32)). The diagram also incorporates a synopsis of recent literature reports. License has been taken to select mechanisms which appear most likely in the light of current knowledge; and features of the pathway of HeLa cell ribosome formation proposed in Fig. III (32) are amplified in the discussion below.

(1) Fingerprinting studies of 80s nucleolar particle proteins

[^{35}S]methionine peptide fingerprints of HeLa cell 80s nucleolar particles (Fig. III (14) A) contain most 50s peptides, but only a few 30s "spots" (see Fig. III (14) C). This result confirms previous preliminary observations that 50s subunit assembly commences at the level of 45s rpreRNA (Maden and Warner, unpublished). In addition, at least some 30s proteins are associated with the 80s nucleolar particle and so it may be

inferred that assembly of 30s ribosomal subunits is initiated in the nucleolus on 80s particles. However, since repeated attempts to isolate 80s material containing all typical 30s peptides have been unsuccessful, and because, by visual observation, 30s material is present in considerably lower molar amounts than 50s peptides, the question arises as to why 30s peptide labelling is not more pronounced.

Cross-contamination of 80s with 55s material (containing 50s peptides - see Fig. III (14) B and Section IV B. (2)) would in itself result in a slight to moderate excess of 50s over 30s peptides, and such contamination has already been noted; on average, 25-30% radioactivity in "80s" material isolated on sucrose gradients is 55s contaminant (Expts. III A.(4), III C. (1) and C. (2)). If 30s and 50s peptides existed in equimolar amounts on pure 80s particles, the above level of contamination would result in a 30s/50s molar ratio of proteins of approximately 3/4.

Expt. III B.(1) showed that in fingerprints of 30s/50s protein mixtures of molar ratio 1/3, all characteristic 30s peptides were distinguishable. Therefore, it follows that the marked deficiency of 30s peptides on the 80s fingerprint does not simply result from the masking effect of contaminant 55s material.

The problem therefore resolves into the selection of one of two possibilities. 80s particles may be deficient in 30s peptides as detected by the two dimensional fingerprinting technique because particle isolation and purification results in selective 30s protein loss. Alternatively, a real deficiency of 30s relative to 50s proteins may exist in 80s particles <u>in vivo</u>.

(a) <u>Degradation of 80s material during purification - a possible cause of 30s peptide deficiency in 80s particles</u>

80s nucleolar particles are unstable and are readily degraded to lower molecular weight material during the isolation procedure (see <u>METHODS</u> (3) b). Indeed, even after isolation, the particles demonstrate an intrinsic instability (Expts. III A. (4) and III E. (4)), but can be stabilised by glutaraldehyde fixation (Figs. III (5) and III (25)).

Unfixed particles, however, on recentrifugation, sediment as two major (80s and 56s) and one minor ribonucleoprotein species (27s). It is probable that the 56s and 27s peaks represent specific "degradation" or "maturation" products of the 80s precursor, and the production of these large fragments of ribonucleoprotein from a single precursor particle is characteristic of endonucleolytic activity as described by Liau et al. (1968) in isolated L cell nucleoli. This specific "degradation" or "maturation" implies that, inherent in the 80s particle is information which dictates the point of scission of the rpreRNA to form its maturation products. A recent report by Mirault and Scherrer (1972) describes a "degradation" process virtually identical to that noted above. These investigators demonstrated that nucleolar 45s RNA-containing ribonucleoprotein particles undergo degradation in vitro to 32s- and 20s-containing ribonucleoprotein products. They also isolated a nucleolar protein fraction containing endoribonuclease activity which accelerates this conversion. However, although specific 80s particle "degradation" during isolation probably makes a significant contribution to the resulting low particle yields noted on extraction of HeLa cell nucleoli, it is doubtful whether such a phenomenon would result in 80s material specifically deficient in 30s peptides. Rather, any degradation of the 80s particles would give products of significantly lower sedimentation coefficient (found to be 56s and 27s - Fig. III (5)) which would not therefore be present in the "80s fraction" sampled from sucrose gradients. Consequently, it is improbable that semi-specific degradation of the 80s particle during its isolation contributes to the relative deficiency of 30s peptide material found on 80s peptide fingerprints.

The results shown in Fig. III (14) D provide a different argument suggesting 80s particle degradation as a possible cause of selective loss of 30s peptides from this nucleolar particle. Spots with mobilities identical to 30s peptides are identifiable in the supernatant of sucrose gradients used to prepare nucleolar particles. This finding may be taken to suggest

that these proteins were "stripped" from 80s particles during or prior to centrifugation. This possibility is very difficult to substantiate, and there is no easy way of determining whether the supernatant proteins were, in fact, "stripped" or indeed were never associated with RNA, but existed as a 30s nucleolar protein "pool".

(b) The 80s nucleolar particle may lack 30s peptides in vivo

An entirely different possible explanation for the relative lack of 30s peptides is that not all of the 30s proteins are assembled into the 80s particle in vivo, some being added to the nascent 30s subunit after separation from its 80s precursor. It has already been noted (Willems et al., 1969), by experiments in which HeLa cell protein synthesis has been inhibited by cycloheximide, that the intracellular "pool" of 30s proteins appears to be considerably smaller than that of their 50s counterpart. Furthermore, it has been inferred from studies involving partial or complete inhibition of protein synthesis in cultured cells (Willems et al., 1969; Maden et al., 1969; Craig and Perry, 1970; Pederson and Kumar, 1971), that the rate at which 45s RNA is cleaved to 32s (+20s) RNA in the nucleolus is closely related to the rate of supply of ribosomal proteins. It is conceivable that, under conditions of normal growth, the supply of 30s proteins limits the assembly of the 80s particle, and that cleavage occurs as soon as the 30s proteins are assembled on the particle. By this reasoning, it is apparent that the majority of 80s particles in the nucleolus would be deficient in 30s proteins. Support for this suggestion derives from kinetic studies of 80s nucleolar particle maturation. Pederson and Kumar (1971) showed that newly synthesised protein continues to be added to pre-existing 80s nucleolar particles, even when 45s rpreRNA transcription is inhibited by actinomycin D. This finding may indicate that 80s particle maturation to its products is immediately preceded by, and may even be consequent upon addition of certain proteins to the particle.

In summary, it is clear that two dimensional fingerprints of 80s particles do not demonstrate all characteristic 30s peptides, and those which can be detected are only weakly labelled. This finding may indicate that 30s proteins are "stripped" selectively from the 80s particle during its purification. Alternatively, the 80s particle *in vivo* may be deficient in 30s proteins. Indirect evidence has been presented to support both views. We consider, in the light of present knowledge, that the latter possibility is more likely.

(2) Two dimensional fingerprinting studies of the 55s nucleolar particle

A number of workers (Warner and Soeiro, 1967; Wu *et al.*, 1971; Kumar and Warner, 1972) have demonstrated by polyacrylamide gel electrophoresis that HeLa cell 55s nucleolar particles contain most proteins found on 50s ribosomal subunits, and kinetic analysis has revealed a precursor-product relationship between these particles (Warner and Soeiro, 1967). The two dimensional protein fingerprinting technique has substantiated these findings. Fig. III (14) (B) and (C) shows that the peptide patterns of ribosomal 50s and nucleolar 55s particles are essentially similar. All rapidly labelled 55s particle peptides are also evident on 50s ribosomal subunits after longer labelling periods (cf. Figs. III (14) B and III (9) A). The "pulse-labelled" 55s particle pattern does not show characteristic 30s peptides (Fig. III (10) B) or additional non-ribosomal peptides, although buoyant density studies suggest that the relative protein content of this particle is higher than that of its 50s product (see Expt. III A. (5) and Section IV B (5)). Moreover, a few characteristic 50s peptides are absent from the 55s particle peptide "map" (e.g. L16, L17). The possible significance of this finding is discussed later (Section IV B. (3)). Confirmation of the precursor-product relationship between the proteins of nucleolar particles and cytoplasmic ribosomes was obtained by a "pulse-chase" experiment. Various aspects of this experiment will now be discussed.

(3) "Pulse-chase" fingerprinting studies of nucleolar particles and cytoplasmic ribosomes.

Experiment III B. (5), a kinetic study of HeLa nucleolar and cytoplasmic ribonucleoprotein particles using the two dimensional fingerprinting technique, confirmed the postulated precursor-product relationship (Section IV B. (1) and (2)) between these particles. All radioactive peptides found on 80s and 55s particles can be "chased", respectively, onto (50s + 30s) and 50s cytoplasmic ribosomal subunits, indicating that all rapidly labelled nucleolar particle proteins are destined to appear on cytoplasmic ribosomes. Analysis of the rate of transit of radioactive protein from nucleolar particles to mature ribosomal subunits shows that this occurs in parallel with the flow of RNA label through these species (see Girard et al., 1965; Greenberg and Penman, 1966). Fig. III (16) demonstrates that most small subunit proteins become assembled into mature 30s cytoplasmic ribosomes within 30 minutes of synthesis, while the majority of 50s proteins do not appear on their respective subunit until after 60 minutes. The fact that some 30s subunit peptides appear to be labelled more slowly than the rest cannot yet be explained, but may reflect different "pool" sizes of individual 30s ribosomal proteins. Likewise, the slower appearance of newly synthesised proteins on the 50s cytoplasmic subunit can be explained in part by the suggestion (Willems et al., 1969) that the 50s protein "pool" is larger than its 30s counterpart. However, this is not the sole explanation, since newly synthesised 28s rRNA also appears more slowly in the cytoplasm than does 18s rRNA (Girard et al., 1965; Penman et al., 1966; Greenberg and Penman, 1966); and the slower migration of nascent 50s subunits through the nucleus is reflected in a preponderance of these particles over 30s nascent ribosomes in nucleolar extracts (Vaughan et al., 1967). The reason for the longer half life of 50s precursor material in the nucleus is unclear. A similar phenomenon is seen during the maturation of E.coli ribosomes. In this organism, maturation of large and small ribosomal subunit precursors is arrested

at several distinct stages, so that intermediate pre-ribosomal particles are isolable (Britten and McCarthy, 1962; Osawa, 1965; Mangiarotti et al., 1968; Sells and Davis, 1970). As in the eukaryotic system, postulated causes of this holdup are purely speculative, but Mangiarotti et al. (1968) have suggested that delay in particle processing may result if the synthesis of a rare component such as 5s RNA (Rosset et al., 1964) or an infrequently occurring protein species becomes rate limiting, or if accretion of the next element in biosynthesis results from a rate limiting transition of the precursor components (RNA and/or protein) from one configuration to another. In vitro reconstruction studies and analysis of cold sensitive mutants favour the latter suggestion (Nomura, 1970) and similar reasoning may explain the delayed appearance of the 50s nucleolar particle in the cytoplasm of HeLa cells.

An important finding of the "pulse-chase" experiment relates to the fingerprint of 50s proteins obtained after a 30 minute "pulse". This peptide "map", unlike an equilibrium-labelled 50s fingerprint, contains approximately 9-12 prominent peptides (Fig. III (16) E), some of which, at least, do not appear on the 80s and 55s "maps". These unusual features were also apparent on the fingerprint obtained after one hour of labelling, but by three hours, a normal equilibrium-labelled pattern was found. From a knowledge of the kinetics of appearance of new ribosomes in the cytoplasm, it was considered likely that the highly labelled peptides isolable from the 50s ribosomal subunit after a 30 minute "pulse" of label represented the cytoplasmic "exchangeable" proteins originally described by Warner (1966) and Warner and Soeiro (1967). This speculation was substantiated by the results of Expt. III B. (6). Fig. III (17) shows that the unusual 50s fingerprint pattern found after a 30 minute "pulse" of radioactivity (Fig. III (16) E) is also obtained when the ribosomes are labelled under conditions which inhibit new ribosome formation. That is, these labelled peptides appear to attach to pre-existing cytoplasmic 50s ribosomes; and

this attachment seems not to be fortuitous, since the "exchangeable" proteins resist the "stripping" effect of a 0.85M KCl wash (Expt. III B (6)). The particle-protein interaction is specific for the 50s subunit, and at least some of those peptides which are added to the 50s subunit in the cytoplasm are not apparent on the 80s and 55s nucleolar particle fingerprint patterns (cf. Figs. III (14) A and B with III (17)). No interchange occurs between small ribosomal subunits and cytoplasmic proteins (Fig. III (17) C).

Finally, it should be noted that cytoplasmic "exchangeable" proteins do not appear as prominent peptides on an equilibrium-labelled 50s fingerprint. A variety of reasons, including those cited in Section IV A. (4) may be invoked to explain this result.

(4) Further kinetic studies demonstrating additional intermediates in the nucleolar particle maturation sequence

Compelling evidence now relates 45s and 32s rpreRNA as precursor and product (Penman et al., 1966; Greenberg and Penman, 1966), and 41s RNA has been shown to be a transitory intermediate in the 45s to 32s RNA conversion step (see Fig. I (1)). Similarly, analysis of nucleolar particles after a "pulse" of [^{14}C]uridine demonstrates a flow of radioactivity from the 80s particle (predominantly labelled at 15 minutes) to the 55s (predominantly labelled at 45 minutes - see Expt. III D. (1)). Moreover, after a 15 minute labelling period, an intermediate ribonucleoprotein particle of sedimentation coefficient 70s was reproducibly evident in very small yield on the sucrose gradient (see inset Fig. III (20) B). After a 30 minute "pulse", radioactive 55s material masked the 70s intermediate (Fig. III (20) B). A similar 70s intermediate was demonstrated more definitively by Mirault et al. (1971) using exponential gradient polyacrylamide gels. This particle contained 41s RNA. They also refer to the isolation of a 40s ribonucleoprotein particle from which was extracted 20s rpreRNA. Since the proteins of these newly described particles have not been investigated, nor their protein/RNA ratios

determined, their status in the ribosome maturation sequence of Fig. III (32) is not firmly established. However, the RNA species found in these particles suggests that the 70s ribonucleoprotein is a derivative of the 80s particle, and itself undergoes maturation to form the putative 40s intermediate and the 55s nucleolar particle. In Fig. III (32), the 70s and 40s particles have been shown, tentatively, to exchange proteins with the nucleolar ribosomal protein pool, and also to release, during maturation, non-ribosomal proteins for recycling through subsequent nucleolar particles. Evidence for these particle-protein interactions is at present speculative, although present information suggests that the nascent 40s nucleolar intermediate, and possibly also its 30s nucleolar product (Expt. III D. (4); Vaughan et al., 1967) is deficient in protein. Presumably, sequestration of 30s proteins from the nucleolar ribosomal protein "pool" is essential for 40s and/or 30s particle maturation (Fig. III (32)).

In contrast to the sequential flow of uridine label from 80s to 55s particles (Fig. III (20) B), nucleolar particle proteins do not show the same consecutive labelling pattern. It appears that after very short incubation periods with [^{3}H]leucine, the 80s particle is the predominantly labelled species. However, this feature is rapidly obscured by incorporation of radioactivity into the 55s particle, so that, at 15 minutes, both nucleolar particles are extensively labelled (Fig. III (20) A). This unexpected result implies that radioactive proteins appear on pre-formed 55s particles (after short labelling times, negligible amounts of newly synthesised 32s RNA have appeared - Fig. III (20) B). Moreover, since the 80s particle has a full complement of rapidly-labelled 55s proteins (cf. Figs. III (14) A and B), and since isopyknic banding of glutaraldehyde-fixed particles (Fig. III (7); Table III (3)) reveals a net loss of protein during the 80s to 55s transition, the kinetic studies would imply that there exists, in the nucleolus, a mechanism whereby proteins are

interchanged between the nucleolar "pool" and the 55s particle. Pederson and Kumar (1971) reached the same conclusion, and also demonstrated that 80s particles become labelled with [^{3}H]leucine even when de novo 45s rpreRNA synthesis is inhibited by actinomycin D, confirming that these particles also participate in protein exchange with the nucleolar "pool".

(5) Buoyant density studies of nucleolar particles.

Liau and Perry (1969) first demonstrated that the nucleolar precursors of ribosomal subunits have a lower buoyant density in CsCl than their cytoplasmic products. Corroborative evidence for this phenomenon has been obtained in the present work (Expt. III A (5)) and also by other investigators (Pederson and Kumar, 1971; Mirault et al., 1971; Kumar and Warner, 1972). The results indicate (Figs. III (6) and (7); Tables III (2) and (3)) that ribosomal precursor particles undergo loss of some protein component during maturation. Since 80s and 55s nucleolar particle maturation involves degradation of approximately 50% and 20% of the particle RNA species, the absolute amount of protein lost from the particles during the maturation process must be considerable, and it has been suggested that both nucleolar particles contain, in addition to ribosomal protein, other polypeptides which remain permanently in the nucleolus (Kumar and Warner, 1972), and which may be associated with non-ribosomal regions of rpreRNA. Kumar and Warner (1972) have studied the non-ribosomal precursor particle proteins extensively and have shown that they are distinguishable from 50s subunit proteins both on the basis of size and labelling kinetics. The proteins, compared with ribosomal proteins, are of high molecular weight, become labelled slowly, and appear to recycle through successive nucleolar precursor particles undergoing processing to mature ribosomal subunits. Evidently, this material is not revealed in the "pulse-labelling" experiments (III B (4) and (5)), since all proteins labelled on nucleolar particles under these conditions can be "chased" onto cytoplasmic ribosomes. It is possible that by labelling

cells for long periods (24-36 hours), [^{35}S]methionine peptide mapping might provide some indication of the complexity of this material. Preliminary experiments have been performed with this object in view, but so far insufficient material has been obtained to permit adequate fingerprinting of the non-ribosomal nucleolar particle proteins. Non-ribosomal nucleolar particle proteins are designated "o" in Fig. III (32) and are shown to become associated exclusively with 80s material and appear in 55s particles by the 80s to 55s maturation process (Fig. I (2)). There is no reason, however, to assume that some of the proteins cannot interact de novo with nucleolar 55s particles, although there is a net loss of particle protein during the 80s to 55s transition (see Table III (3)).

(6) Cytoplasmic maturation of newly formed ribosomes.

The final step of eukaryotic ribosome maturation seems to be cytoplasmic (Section I C. (2) (c)), and Perry and Kelley (1966a), and Chen et al. (1971), working independently, have demonstrated that this terminal step involves modification of the newly formed subunits in the cytoplasm. The subunits lose low density material (presumably protein, but possibly also lipid) to become ribonucleoprotein particles of higher buoyant density (i.e. lower protein/RNA ratio). The maturation process is completed within two hours of particle formation (Perry and Kelley, 1966a). Another modification of cytoplasmic 50s subunits has already been described (Section IV B. (3)). This modification differs from that observed by Perry and Kelley (1966a) and Chen et al. (1971) and involves protein exchange between the cytoplasmic protein "pool" and mature 50s ribosomal subparticles (Expt. III B. (6)). A similar particle-protein interaction in E. coli ribosomes was noted by Kurland et al. (1969), who showed that the protein exchange is associated with an enhanced activity in protein synthesis. Consequently, these workers have proposed, tentatively, that the structure of the ribosome is modified

during the various stages of protein synthesis. A similar phenomenon in HeLa cells would explain the cytoplasmic 50s subunit-protein interaction which was noted in Expt. III B (6) and by Warner and Soeiro (1967).

Finally, it is of interest to compare the different classes of ribosomal protein described in this study with those detailed by Warner (1966). He distinguishes three types of protein which are associated with HeLa cytoplasmic ribosomes.

Class A proteins

These enter the cytoplasmic ribosomes only in the company of newly formed rRNA, and would appear to correspond to the structural ribosomal proteins which, in this study, could be isolated from 80s and 55s nucleolar particles.

Class B proteins

are specific cytoplasmic polypeptides which appear to exchange with ribosomal proteins, but remain firmly bound to the ribosome after extraction from the cell. Similar "exchangeable" cytoplasmic proteins have been fingerprinted in this study.

Class C proteins

apparently associate non-specifically with cytoplasmic ribosomes and can be removed from them at ionic strength 0.2. Such proteins were found in this study not to contribute significantly to the cytoplasmic ribosomal protein complement.

Conclusion

Initiation of small ribosomal subunit formation in HeLa cells involves interaction of at least some 30s proteins with the 45s rpreRNA of 80s nucleolar particles. The fact that not all 30s proteins are found on isolated 80s particles may result from particle degradation during the isolation process, or may reflect the in vivo state of these particles. 80s nucleolar particles also contain all rapidly labelled peptides present on 55s nucleolar ribonucleoprotein complexes,and these can be "chased" from both nucleolar precursors into cytoplasmic 50s subunits. There is, from buoyant density data, a net loss of protein from the nucleolar particles as they mature to cytoplasmic ribosomal subunits, and kinetic experiments indicate that the maturing precursor particle proteins undergo exchange with a nucleolar protein "pool".

30s subunits become more rapidly labelled with radioactive amino acids than do their 50s counterpart, but a few 30s proteins are labelled more slowly than the remainder. After a 30 minute labelling period, the 30s fingerprint resembles an equilibrium labelled pattern. Conversely, the "map" of 50s subunit peptides, isolated 30 minutes after commencement of labelling is not typical of the equilibrium pattern found at three hours, but presents a characteristic fingerprint of 9-12 strongly labelled peptides. Since most of these peptides are not identifiable on 80s and 55s fingerprints, but are isolable from 50s subunits labelled in the presence of actinomycin D or toyocamycin (both inhibit new ribosome formation), it has been concluded that they represent proteins which "exchange" with pre-existing 50s subunits in the cytoplasm. No similar exchange occurs with 30s ribosomal subunits.

Finally, a previously unrecognised 70s particle is described, and present understanding of non-ribosomal nucleolar particle proteins is discussed in the light of buoyant density studies.

The results have been summarised in diagrammatic form (Fig. III (32)).

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Summary of Thesis presented for Degree

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A study of the proteins of nascent and mature mammalian ribosomes

Several studies on mammalian cell ribosome structure and formation have utilised one dimensional protein separation techniques such as gel electrophoresis to fractionate ribosomal proteins. Such procedures have provided information of considerable value, but nevertheless suffer from limitations. Proteins from the small ribosomal subunit are not clearly resolved from those of the large subunit, and fractionation is based on gross properties of the proteins such as size or charge, rather than on detailed chemical properties arising from the amino acid sequences of the protein.

In this report are presented results from an analysis of HeLa cell ribosomes and their nucleolar precursor particles using a methionine peptide mapping technique. Cells were labelled with L-[^{35}S]methionine, ribosomal subunits or nucleolar ribonucleoprotein particles prepared, their proteins digested with trypsin without further prior fractionation, and the digestion products fingerprinted on paper. The advantages of a convenient two dimensional fractionation procedure were thereby combined with those of exploiting detailed chemical features of the proteins. The use of radioactive methionine resulted in selective labelling of a restricted number of peptides. This simplified the fingerprints and allowed "pulse-chase" and related kinetic experiments to be undertaken.

Selective labelling of peptides with sulphur-containing amino acids was also used in a previous study of Escherichia coli ribosomal proteins. From its use in the present project, the procedure provided the following information: First, HeLa cell large and small ribosomal subunit proteins possess major structural differences from each other. These differences are apparent even when modification is made to the method of ribosome preparation or of protein treatment prior to fractionation. Also, the degree of similarity between ribosomal proteins derived from different species was found to depend on the evolutionary relationship which exists between the species. Secondly, by analysis of nucleolar ribonucleoprotein particles, it was

found that the bulk of HeLa cell 50s ribosomal assembly is completed in the nucleolus on 55s particles which contain the 32s precursor of ribosomal 28s RNA. However, a small amount of specific exchangeable protein is added to the large ribosomal subunit in the cytoplasm, and this addition is not inhibited by drugs which prevent new ribosome formation.

Thirdly, both large and small subunit assembly commences on the 45s RNA of 80s nucleolar particles, although small subunit protein representation on the isolated 80s precursor is incomplete, and those proteins which are present are found in much less than molar amount. This phenomenon may be a true representation of the structure of the 80s particle in vivo or may result from the procedure used to isolate the particle. However, modification of the method of 80s particle extraction did not improve the yield of 30s proteins found on that precursor.

Using other techniques, further information was obtained regarding the assembly of animal cell ribosomes. Kinetic analysis of the nucleolar particles revealed an exchange of protein between these particles and a nucleolar protein "pool". Moreover, a nucleolar 70s particle was found to exist as a transient intermediate in the 80s to 55s particle conversion step. Finally, from buoyant density data, it was inferred that there is sequential protein loss from the nucleolar particle precursors during their maturation to cytoplasmic ribosomal subunits. These proteins which are lost appear to be exclusive to the nucleolar particles and are not found as structural components of cytoplasmic ribosomes.

Correlation of the above information with data from other sources has permitted the formulation of a tentative mechanism of ribosome formation in animal cells.